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TITLE: Validation of Quantitative Multimodality Analysis of Telomerase Activity in  
Urine Cells as a Noninvasive Diagnostic and Prognostic Tool for Prostate  
Cancer

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14. ABSTRACT <i>The major goal</i> of this project was to develop a clinical trial focused on the validation of diagnostic and prognostic utility of <i>quantitative</i> analysis of telomerase activity in exfoliated cells in urine as a molecular marker and clinical tool for: 1) noninvasive early detection of prostate cancer; and 2) determination of indolent asymptomatic carcinomas that have potential to develop into invasive disease and require treatment. We report here that our research and clinical teams are completely ready to carry out the proposed clinical trial. We have developed and optimized all research and clinical protocols. We have prepared all material resources for large-scale complex analysis of telomerase activity in patients exfoliated cancer cells. We have established a reliable crosscommunication between our clinical and research personnel.					
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## Introduction

**The major goal** of this project was to develop a clinical trial focused on the validation of diagnostic and prognostic utility of *quantitative* analysis of telomerase activity in exfoliated cells in urine as a molecular marker and clinical tool for: 1) noninvasive early detection of prostate cancer; and 2) determination of indolent asymptomatic carcinomas that have potential to develop into invasive disease and require treatment.

We have proposed this clinical trial as a logical continuation of our study, supported by the DOD Idea Award (PCRP03), which was focused on the development of a new data acquisition system for telomerase activity analysis.

The urgent necessity for the translation into clinical use a noninvasive yet more sensitive and specific assays for prostate cancer screening and monitoring in general, and for the proposed clinical trial in particular, is explained by the current status of the field:

- Prostate cancer is a second leading cause of cancer death among men and the most common malignancy (Jemal et al., 03; Kosary et al., 95) that affects about 30% of young men, and 64% of men 60 to 70 years of age (Sakr et al., 93; 94; Walsh, 94), and requires an annual screening for all men after 40 years of age.
- By the end of 2004, more than 230,000 men in the United States were diagnosed with prostate cancer, and about 30, 000 died of the disease (Report to the Nation on Prostate cancer, 2004; Jemal et al., 2004).
- Furthermore, a dramatic number of new cases is expected in the nearest decade, because the “baby boomer” men will reach the target age for prostate cancer.
- There is a crucial necessity not only to detect prostate cancer, but also to distinguish between the most aggressive carcinomas that carry the potential to develop into lethal disease and need more aggressive treatment, and indolent carcinomas that require monitoring.
- Prostate cancer is a heterogeneous and multifocal disease with usually low malignant cell infiltration that significantly complicates a collection of representative tumor samples subjected for standard clinical analysis.
- At present time, ***none of existing standard screening methods are sufficiently sensitive and/or specific for noninvasive, early, large-scale prostate cancer screening.***
- There is a lack of satisfactory tools for reliable prediction of the invasive potential of indolent carcinomas.

## Body

### Approved Objectives

To provide a foundation for successful execution of this goal, we have to undertake the following steps during the proposed period:

- I. Establish and secure reliable and continuous recruitment of patients by

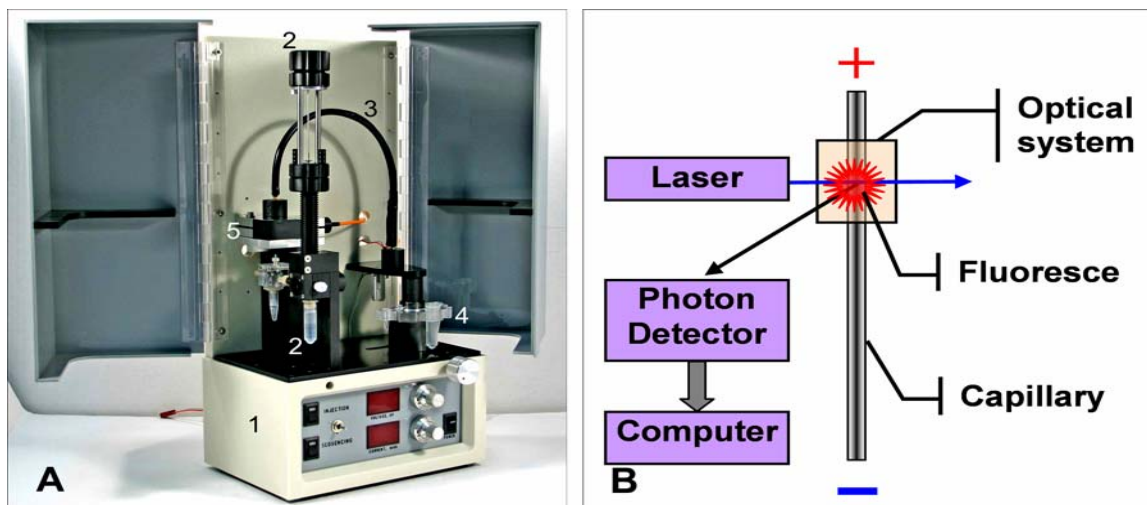
- developing website and brochure with information about the proposed clinical trial, and providing educational lectures and seminars at participating sites.
  - establishing collaboration with several urologists in the VA Northport, NY;
  - further developing our existing collaboration with the Department of Urology, SUNYSB.
- II. Develop uniform clinical protocol and consent form.
- III. Develop necessary material resources for the proposed clinical trial by a) providing the laboratory of Dr. Botchkina with additional equipment to serve as a *central core and a foundation* for the proposed clinical trial, and b) purchasing of the necessary equipment for primary sample processing at each participating clinical site to secure a quality of samples.

We report here that our research and clinical team is completely ready to carry out the proposed clinical trial.

We have demonstrated that analysis of telomerase activity in exfoliated cells in urine has 100% sensitivity for prostate cancer and, therefore, has a high potential as a molecular marker for early detection of this malignancy (Botchkina et al., 05).

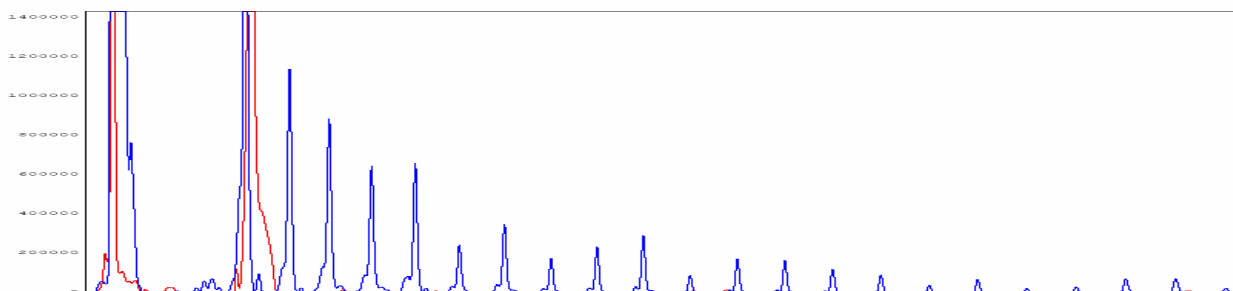
Our data on *quantitative* comparative analysis of telomerase activity in different prostate cancer cell lines have revealed that cells with higher malignant potential express much higher telomerase activity (Botchkina et al., 04). Our data on *quantitative* measurement of telomerase activity in exfoliated cells from urine of men with clinically confirmed benign prostatic hyperplasia (BPH) during routine prostate cancer screening, have shown differential levels of telomerase activity (Adler et al., 04). Therefore, we believe that the proposed assay will allow the identification of indolent and aggressive carcinomas.

We believe also that the combined use of a quantitative real-time PCR and capillary electrophoresis analysis of telomerase activity by Stony Brook Telomerase Analyzer (see Figure 1) as a back up control for cases with low or negative telomerase activity should additionally improve sensitivity and specificity of the proposed assay.



**Figure 1.** The bench-top prototype of **SBTA** originates from single photon detection-based DNA sequencer. A: general view. 1 - high voltage supply with a built-in voltmeter and amperemeter; 2 - polymer replacement system; 3 - temperature control system; 4 - tube-changer carousel; 5 – fiberized precision optical system. B: schematic principle of the instrument.

The SBTA should be very important clinical tool allowing avoid possible false-positive and false-negative measurements of telomerase activity since it can detect and analyze highly specific 6 base pairs fragments of telomerase activity product (see Fig. 2).



**Figure 2.** Color-deconvoluted electropherograms of samples containing negative control (A),  $10^2$  cells + ILS (B), and  $10^2$  cells from optimized injection experiment (C) after real-time PCR with TRAPeze XL kit. Traces of red-fluorescing material are depicted here and below in red color, green fluorescing – in blue.

Large statistical analysis of correlations between accurately evaluated levels of telomerase activity and standard clinicopathological data will allow us to make a conclusion about clinical utility and applicability of the proposed assay as a diagnostic and prognostic marker for prostate cancer. The proposed assay is *noninvasive*, so it not only presents *no potential risks*, and requires *no additional efforts or medical cost*, but it also allows multiple repeated

tests that can have a major impact on early prostate cancer screening and monitoring, improving the sensitivity and specificity of testing.

## KEY RESEARCH ACCOMPLISHMENTS

Not Applicable (*No research* was required in this proposal).

## REPORTABLE OUTCOMES

1. We have submitted our Proposal and Clinical Protocol for *Clinical Trial Award* (CTA, DOD PCRP 04) as a logical continuation of the current project.
2. We have developed a reliable way for patient accrual/recruitment, including the IRB approved Consent form and brochure.
3. We have prepared all equipment and material resources for large-scale complex analysis of telomerase activity in patients exfoliated cancer cells.
4. We have established a reliable cross-communication between our clinical and research personnel.

## CONCLUSIONS

Our team is completely prepared for the proposed clinical trial which is urgently needed for the improved prostate cancer screening and monitoring.

We are looking forward to resubmit our proposal to the DOD CTA in the nearest future because we strongly believe that the proposed assay can be a highly valuable clinical and research tool.

## PUBLICATIONS

Adler HL, Frischer ZI, Kirshenbaum A, Kim RH, and Botchkina GI. Real-time PCR (RTQ-TRAP) analysis of telomerase activity in urinary cellular content of prostate cancer patients. *Proceedings of the AACR/JCA "Advances in Cancer Research"*, January 2004.

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## APPENDIX

1. Paper in *Clinical Cancer Research*, 2005
2. Paper in *Biosensors*, 2005 (in press)
3. Abstract 1 (AACR/JCS)
4. Abstract 2 (AACR)
5. Abstract 3 (AACR)
6. Abstract 4 (NIH)
7. Newsday article (July 2005)
8. SUNY article (August 2005)

## **Noninvasive Detection of Prostate Cancer by Quantitative Analysis of Telomerase Activity**

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**Abstract** **Purpose:** Prostate cancer is the most common male malignancy and the second leading cause of male cancer death; therefore, there is urgent necessity for noninvasive assays for early detection of prostate cancer. Obtaining prostate tumor samples surgically is problematic because the malignancy is heterogeneous and multifocal and early-stage tumors are nonpalpable. In contrast, exfoliated cells represent the cancer status of the entire gland better due to the general tendency of cancer cells to exfoliate into biological fluids. The purpose of this study was to clarify whether quantitative analysis of telomerase activity in exfoliated cells in urine could serve as a reliable molecular marker of prostate malignancy.

**Experimental Design:** We analyzed prospectively post-prostatic examination – exfoliated cells from the urine of 56 patients undergoing routine prostate screening. Epithelial cells were isolated and enriched by immunomagnetic separation. Telomerase activity was analyzed by quantitative real-time PCR telomeric-repeat amplification protocol assay using Opticon MJ research instrument.

**Results:** We report now that all prostate cancer patients revealed high levels of telomerase activity thereby showing 100% of the assay sensitivity. In contrast, the majority of patients with clinically confirmed benign prostatic hyperplasia (BPH) did not express any telomerase activity (70% of all BPH patients), most likely presenting cancer-free cases, or expressed low levels of activity (18%). However, about 12% of BPH patients revealed high levels of telomerase activity that potentially can reflect hidden prostate cancer.

**Conclusions:** We suggest that the quantitative analysis of telomerase activity can be useful for the selection of prostate cancer and cancer-free cases.

Prostate cancer is the most common male malignancy and the second leading cause of male cancer-related death (1, 2). Small prostatic carcinomas were detected in 30% of men ages 30 to 49 years, 40% of males over age 50, and 64% of men ages 60 to 70 years (3–5). For this reason, an annual screening is generally recommended for all men over the age of 50. Screening on such a large scale requires noninvasive methods of detection and the use of reliable, clinically validated markers of prostate cancer. On the other hand, the total number of detected carcinomas exceeds the number of clinically manifested cases; therefore, there is a need for the markers that are suitable not only for distinction between benign and malignant growth but also for the selection of potentially aggressive tumors that require more aggressive treatment.

Prostate cancer screening is currently based on the digital rectal examination (DRE), prostate-specific antigen (PSA) monitoring, and transrectal ultrasound (TRUS) with TRUS-guided biopsy for morphologic examination as the reference standard. However, because prostate cancer is a heterogeneous and multifocal disease with typically low malignant cell infiltration, obtaining the representative tumor samples by biopsy is problematic. None of the standard methods are sufficiently sensitive and specific for prostate cancer, which makes early detection of the disease difficult. Early-stage prostate tumors are nonpalpable by definition, and about one third of all prostate tumors are not accessible to the DRE by location. PSA elevation (the recommended threshold levels for further diagnostic evaluation are  $\geq 4.0$  ng/mL) is not specific for cancer because PSA is also produced by other tissues and it occurs in men with benign diseases or after physical or surgical manipulation of prostate; in addition, men with prostate cancer often have normal PSA levels (5–7). A TRUS-guided needle biopsy may miss up to 34% of clinically localized prostate cancers, and about 10% to 19% of patients with initially negative needle biopsy were diagnosed with prostate cancer on a second biopsy (8–10). The morphologic manifestation and the expression of some molecular markers in multiple tissue samples obtained from different areas of cancer-bearing prostates vary dramatically (11); thus, each biopsy can miss the presence of prostate cancer, including even the most aggressive foci.

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In contrast to invasive sampling, prostate manipulation during DRE can harvest a representative pool of prostate cancer cells, as was shown earlier (12–14). This is possible because of the increased ability of cancer cells compared with normal cells to exfoliate into biological fluids (15) due to the weakening of cell-to-cell and cell-to-extracellular matrix contacts as a result of up-regulation and activation of extracellular matrix-degrading enzymes (16, 17). Exfoliated prostate cancer cells can be isolated from urine because about 3 to 4 cm of the urethra lies entirely within the prostate and receives the openings of all ducts of the gland. Indeed, previous data have shown not only the presence of viable tumor cells harvested by prostate massage in urine specimens from prostate cancer patients but also a significant correlation of their biological variables with the histopathologic status of the same surgically removed tissues (13, 14). The proportion of prostate cells among epithelial cells present in urine samples is higher in men with prostate cancer than in men who are free of prostate cancer (18). In general, the attempts to detect these cells by routine urine cytology are not expedient due to relatively low number of malignant cells, especially at the early stages of prostate cancer and high false-negative rates (up to 50%) of the method (19–21). In contrast, modern PCR-based molecular methods can detect much lower numbers of malignant cells compared with conventional histologic or cytologic examination. In addition, these methods are free of subjective data interpretation and have a high throughput, thereby providing a reliable basis for noninvasive cancer detection upon the availability of specific molecular markers of cancer.

Recent widespread interest in telomerase was initiated by the discovery that unlimited proliferation in most cancer and immortal cells is highly dependent on the activity of this ribonucleoprotein enzyme complex (22). Telomerase has the essential biological function of protecting DNA from degradation by catalyzing the elongation of telomeres by addition of noncoding repeated TTAGGG sequences to the ends of chromosomes (23). In contrast to almost 100 proposed molecular markers that can be applied only to a single or several types of cancer, telomerase is unique due to its almost universal expression in a vast majority of tumor tissues and its general lack of expression in normal somatic tissues, with the exception of highly proliferative tissues, germ line, and stem cells (24, 25). Telomerase activity may serve as a useful marker for early cancer detection because in many cancer types it appears early in the preneoplasia state (26). The diagnostic and prognostic utility of telomerase has been widely studied. There is increasing evidence that stronger telomerase activity with higher rate of detection correlates with poorly differentiated cancers and higher Gleason scores (26–29). It was suggested that the levels of telomerase activity might predict clinical outcome because high levels usually correlate with poor prognosis and higher cancer aggressiveness (30). The value of telomerase as a diagnostic marker for one particular urologic malignancy, bladder cancer, has been addressed in a large number of studies (reviewed in refs. 21, 31). Recent comparative analysis of various screening methods has shown that telomerase activity has the highest combination of sensitivity and specificity for cancer diagnosis reaching sensitivity of up to 90% and clinical specificity for cancer of 94% to 100% (21, 32–35). It is important to note that the sensitivity of telomerase assays at the early stages of cancer development is

significantly higher than the sensitivity of cytology-based assays, 75% versus 8%, respectively (36).

Telomerase is strongly associated with prostate cancer showing activation in up to 93% of cases and in all cell lines derived from human prostate cancer (27, 28, 37–39). In contrast, no telomerase activity was detected in normal uncultured prostate tissues. However, conflicting data were reported about telomerase activity in the adjacent noncancerous tissues. When a malignancy is present, the adjacent tissues may be contaminated by neighboring cancer cells and may show telomerase activity (25). Thus, it was shown that about 10% of samples from benign prostatic hyperplasia (BPH) are telomerase positive (27, 37); however, if the prostate is confirmed to be cancer-free, BPH tissues are telomerase negative (37, 38, 40). Presently, differential detection of benign and malignant prostatic diseases is one of the most challenging problems in the field and it requires clinical validation of molecular markers suitable for noninvasive screening for prostate cancer. One of the most promising candidates for this role is telomerase. However, only limited data exist about the correlation of telomerase activity in exfoliated cells in urine with prostate clinicopathology (35, 41, 42). In addition, accurate quantitative methods of telomerase activity measurements are required for reliable evaluation of the clinical utility of telomerase. Therefore, the major goal of this study was to clarify whether the quantitative analysis of telomerase activity in naturally voided urine specimens collected after DRE could serve as a reliable molecular marker of prostate malignancy. As an attempt to avoid false-positive and false-negative measurements of telomerase activity, we used immunomagnetic isolation of epithelial cells and highly sensitive quantitative real-time PCR telomeric-repeat amplification protocol (TRAP) assay. In addition, to avoid possible overestimation of the clinical utility of telomerase assay, we analyzed all samples prospectively, without prior knowledge of the patients' clinicopathologic status.

## Materials and Methods

**Patients.** Patients undergoing routine medical examination of the prostate at the Department of Urology, State University of New York at Stony Brook and willing to participate in this study by signing the Institutional Review Board–approved consent form were recruited by participating urologists. Careful thorough palpation and examination of the prostate done by urologist during DRE to evaluate its size and presence of tumor was considered as a prostate massage. Naturally voided urine specimens were collected prospectively, immediately after medical examination, without prior knowledge of the patient's clinicopathologic data. The clinicopathologic status of each patient was assessed with the following standard procedures widely used for prostate cancer screening: clinical medical history and physical examination, DRE, serum PSA levels measurement, urine cytology, TRUS examination, and TRUS-guided biopsy for morphologic examination as a reference test. Histomorphologic grading was done in accordance to the Gleason system and staging of prostate cancer was defined according to the international tumor-node-metastasis classification. In all cases, results of standard pathologic study and quantitative measurements of telomerase activity by real-time PCR TRAP assay were determined independently, in a double-blind manner.

**Isolation of exfoliated cells.** Immediately after collection, urine specimens were put on ice and centrifuged as soon as possible (not later than a 20-minute interval) at 3,000 rpm, 4°C, for 7 minutes. Pelleted exfoliated cells were washed twice, first in 10 mL of cold PBS (pH 7.4)

followed by 2 mL of PBS containing 100 units/mL of RNase and protease inhibitor cocktail (SUPERase-In; Ambion, Palo Alto, CA).

**Immunomagnetic cell sorting.** To enrich and isolate malignant and normal epithelial cells from other cells in urine sediment, we used colloidal superparamagnetic microbeads conjugated with monoclonal antibodies against human epithelial antigen and the magnetic cell sorting device (miniMACS; Miltenyi Biotec, Auburn, CA) as recommended by the manufacturer. Briefly, washed and pelleted exfoliated cells were resuspended in 400  $\mu$ L ice-cold buffer and incubated with 50  $\mu$ L of human epithelial antigen microbeads and 50  $\mu$ L of blocking reagent at 4°C to 6°C on shaker followed by washing with 5 mL cold PBS containing 2 mmol/L EDTA, 0.5% bovine serum albumin, and 100 units/mL of SUPERase. Analyzed sample was placed into miniMACS separation column and the outcoming buffer with *negatively* selected cells was discarded. Then, miniMACS column was removed from magnetic device, washed with 1 mL PBS containing SUPERase, and *positively* selected by immunomagnetic beads epithelial cells were collected for further processing.

**Protein extraction.** Cells were pelleted again at 3,000 rpm, 4°C, for 7 minutes and resuspended in 25 to 50  $\mu$ L of ice-cold CHAPS lysis buffer (Celliance, Norcross, GA) containing 100 units/mL of SUPERase followed by an incubation on ice for 30 minutes. Lysates were then centrifuged at high speed (16,000  $\times$  g for 20 minutes at 4°C). The aliquots of supernatant fluid was aliquoted and stored at -80°C.

**Prostate cancer cell lines.** Human prostate cancer cells, PC-3, were obtained from the State University of New York cell culture/hybridoma facility, where they were routinely cultured. Cells were harvested using trypsin-EDTA (Sigma, St. Louis, MO) and processed as it was described for exfoliated cells. Same protein extract from PC-3 cells was used in each real-time PCR run as a positive control and as a standard. Serial dilutions of protein extracts from these telomerase-positive cells, equivalent to  $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$ , 10, and 1 cell, were used for standardization and relative quantification of telomerase activity in clinical samples by previously described real-time quantitative TRAP assays (42). Modified highly tumorigenic prostate cancer PC3-P and PC3-MM2 cells were kindly provided by Dr. I. Fidler (M.D. Anderson Cancer Center, Houston, TX). The protein concentration was measured using the bicinchoninic acid protein assay (Pierce, Rockford, IL).

**Real-time quantitative telomeric-repeat amplification protocol assay.** Real-time PCR analysis of telomerase activity was carried out in a 96-well plate using the Opticon MJ Research instrument and optimized standard SYBR Green protocol. A fluorescence dye SYBR Green is capable of binding with exceptionally high affinity to the double-stranded amplicons and generating fluorescence signals after each PCR cycle. Collected fluorescence signals were analyzed with the Opticon software. The TS and ACX primers (TS, 5'-AATCCGTCGAGCAGAGT-3'; ACX, 5'-GCGCGGCTTACCCTTACCCTTACCCTAACC-3') were synthesized by Invitrogen Life Technologies (San Diego, CA). Stock solution of each primer (1  $\mu$ g/ $\mu$ L) was aliquoted and kept at -20°C. Total reaction volume was 20  $\mu$ L per well, containing 10  $\mu$ L of 2 $\times$  SYBR Green Master Mix (Qiagen, Chatsworth, CA), 0.1  $\mu$ g of each primer, 2  $\mu$ L of protein extract, and 7.8  $\mu$ L of RNase-free water. The reaction mixture was first incubated at 25°C for 20 minutes to allow the telomerase presented in the protein extract to elongate the TS primer by adding TTAGGG repeat sequences. The PCR was then started at 95°C for 15 minutes (hot start) to activate the AmpliTaq polymerase, followed by a 40-cycle amplification (denaturation at 95°C for 20 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 60 seconds, and plate reading at 60°C for 10 seconds). Fluorescence signals produced by binding of SYBR Green to new double-stranded amplicons were collected and analyzed after each PCR cycle with Opticon software. All samples were run in duplicates. Reaction mixture with 2  $\mu$ L of CHAPS lysis buffer instead of protein extract (no target) was used as a negative control. Serial dilutions of the protein extracts from known number of prostate cancer PC-3 cells were used for construction of the standard curve for relative quantification of telomerase activity in patients samples and as a positive control. Relative telomerase activity

in patient samples was calculated based on the threshold cycle ( $C_t$ ). The measurements of fluorescence signals at the early phase of exponential amplification allows comparative analysis of samples with different initial amount of target sequences and ensures the accuracy of the quantification because at this point the accumulation of inhibitory PCR products and the limitation of the reaction products are unlikely to occur.

**Statistical analysis.** Telomerase activity oriented toward detection of prostate cancer was compared with the standard diagnostic criteria based on clinicopathologic variables. The levels of telomerase activity in patient samples (integrated fluorescence per PCR cycle) were normalized to the levels of activity in the known number of prostate cancer PC-3 cells using a standard curve and Opticon MJ software. The cut point for telomerase assessment was determined to be 100 cell equivalent based on ROC curves. Sensitivities, specificities, positive and negative predictive values, overall percent agreements, and kappa statistics were estimated based on this cut point. All of the above statistics were also estimated for standard PSA measurements. The widely accepted PSA cut point of 4.0 ng/mL was used. Pearson test was done to evaluate correlations between levels of telomerase activity and the clinicopathologic variables in prostate cancer patients and patients with nonmalignant prostate diseases. Sensitivity, specificity, and the predictive values of our measurements was estimated by comparing levels of telomerase activity with the presence or absence of cancer, as well as with the presence or absence of nonmalignant disease, via standard clinicopathologic tests. Kappa statistics were used to assess this association. The Gleason score and presence or absence of telomerase activity was compared using the Mann-Whitney test.  $P$ s < 0.05 were considered statistically significant.

## Results

**Quantitative analysis of telomerase activity.** For each experiment, under optimized conditions, we set up standard curves for serial dilutions of telomerase-competent prostate cancer PC-3 cell extracts, equivalent to different cell count (from 100,000 cells to single cells). Linearity and accuracy of real-time quantitative TRAP assay were tested using the same samples (serially diluted extracts of PC-3 cells) analyzed in 12 separate experiments in duplicates that have shown high reproducibility and precision (a systematic error was 0.121 PCR cycle;  $P$  < 0.001). The limits of sensitivity for the Opticon MJ Research were determined as about 10 PC-3 cells; thus, all measurements of the telomerase activity in patient samples were considered as negative if after normalization to the PC-3 cell equivalent the numbers were <10.

Subsequent review of the coded medical records revealed nine patients with clinically confirmed prostate cancer, one patient with atypical cells suspicious for prostate cancer, two patients with high-grade prostatic intraepithelial neoplasia and 44 patients with no clinical evidence of malignant disease (BPH). Table 1 represents a summary of all cases with standard pathologic evaluation, telomerase activity, and PSA levels measurements. Median patient age was 61.3 years, ranging from 41 to 82 years. After immunomagnetic separation of the epithelial pool of exfoliated cells (that may or may not contain prostate cancer cells) from the rest of urinary cellular content, high levels of telomerase activity equivalent to the average 4,242 cells (range, 100-19,489 PC-3 cells) were detected in all patients with clinically localized prostate cancer ( $n$  = 9; Table 1). Two of two patients with high-grade prostatic intraepithelial neoplasia and one of one patient with atypical cells suspicious for prostate cancer also revealed relatively high levels of

**Table 1.** Summary of all cases for telomerase activity and total serum PSA levels

Case	Pathology	Telomerase activity		PSA (ng/mL)	
		0-100	≥100	≤4	≥4
1	Prostate cancer	—	100	—	6.3
2	Prostate cancer	—	225	—	74.1
3	Prostate cancer	—	235	—	4.2
4	Prostate cancer	—	450	—	21.2
5	Prostate cancer	—	732	—	5.0
6	Prostate cancer	—	3870	—	4.1
7	Prostate cancer	—	5830	—	8.76
8	Prostate cancer	—	7247	3.49	—
9	Prostate cancer	—	19489	—	13.25
10	HG PIN	—	157	—	4.6
11	HG PIN	—	202	—	4.0
12	Atypical Cells	—	214	2.66	—
13	BPH	0	—	2.8	—
14	BPH	0	—	—	7.0
15	BPH	0	—	—	7.0
16	BPH	0	—	NA	NA
17	BPH	0	—	—	8.0
18	BPH	0	—	0.47	—
19	BPH	0	—	1.36	—
20	BPH	0	—	0.97	—
21	BPH	0	—	1.88	—
22	BPH	0	—	0.75	—
23	BPH	0	—	1.22	—
24	BPH	0	—	1.30	—
25	BPH	0	—	1.98	—
26	BPH	0	—	1.48	—
27	BPH	0	—	1.76	—
28	BPH	0	—	—	10.08
29	BPH	0	—	0.31	—
30	BPH	0	—	2.13	—

**Table 1.** Summary of all cases for telomerase activity and total serum PSA levels (Cont'd)

Case	Pathology	Telomerase activity		PSA (ng/mL)	
		0-100	≥100	≤4	≥4
31	BPH	0	—	—	8.39
32	BPH	0	—	0.43	—
33	BPH	0	—	0.14	—
34	BPH	0	—	1.47	—
35	BPH	0	—	2.52	—
36	BPH	0	—	1.33	—
37	BPH	0	—	1.21	—
38	BPH	0	—	1.57	—
39	BPH	0	—	1.22	—
40	BPH	0	—	1.43	—
41	BPH	0	—	0.33	—
42	BPH	0	—	0.38	—
43	BPH	0	—	—	11.3
44	BPH	0 (55)	—	—	6.7
45	BPH	0 (13)	—	1.21	—
46	BPH	0 (19)	—	1.27	—
47	BPH	0 (26)	—	0.96	—
48	BPH	0 (32)	—	0.69	—
49	BPH	0 (53)	—	0.48	—
50	BPH	0 (88)	—	2.57	—
51	BPH	0 (89)	—	—	12.1
52	BPH		164	—	8
53	BPH		173	1.55	—
54	BPH		176	1.15	—
55	BPH		431	—	4.7
56	BPH		616	1.71	—

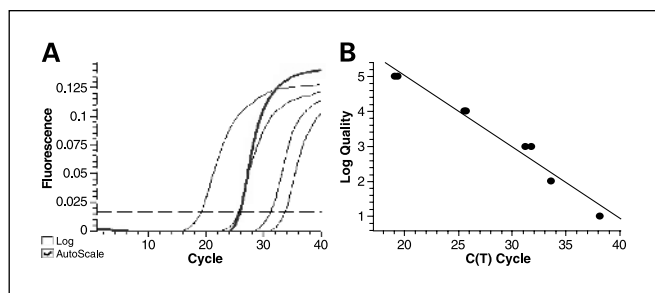
Abbreviations: HG PIN, high-grade prostatic intraepithelial neoplasia; NA, not applicable.

telomerase activity (average 330 cell equivalent for high-grade prostatic intraepithelial neoplasia and 214 for atypia). Figure 1 shows a representative real-time PCR TRAP analysis of telomerase activity in patient with clinically confirmed prostate cancer. Amplification plots revealed a cell number-dependent amplification of telomeric repeats on serial dilutions of PC-3 cells. Possible artifacts (such as primer-dimer amplification) were monitored by using *no template control* by addition of the lysis buffer instead of the protein extract to the reaction mixture. At described conditions, the primer-dimer amplification did not occur because fluorescence signals in negative controls and highly diluted samples were always below the amplification threshold (*dotted line, left*).

The cohort of patients diagnosed with BPH ( $n = 44$ ) revealed three different subgroups based on the levels of telomerase activity normalized to the number of PC-3 cells (see Table 1; Fig. 2, *right*). The first largest group (31 of 44 cases, ~70%) did not show any amplification of the telomeric repeats that was considered as an absence of telomerase activity. The second group (8 of 44, ~18%) expressed low levels of telomerase activity equivalent to the average 46 PC-3 cells (range, 13-89 cell equivalent). The third group (5 of 44, ~12%) showed

relatively high levels of telomerase activity at average 312 PC-3 cells (range, 164-616 cells), comparable with those of patients with clinically localized prostate cancer. One patient diagnosed with BPH and inflammation had relatively high levels of telomerase activity; in about 1 year, he was diagnosed with prostate cancer (Gleason score 7).

**Statistical analysis.** We have evaluated the *sensitivity* (percent of the telomerase-positive cases among cases with clinically localized prostate cancer), *specificity* (percent of the telomerase-negative cases among clinically defined nonmalignant cases), *positive predictive value* (a probability to have clinically localized prostate cancer if telomerase activity is positive), and *negative predictive value* (a probability to be clinically free of prostate cancer if telomerase activity is negative). To evaluate these variables, we have excluded two cases with high-grade prostatic intraepithelial neoplasia and one case with atypical cells as cases with clinically indefinite diagnosis (neither normal nor cancer). We used a standard cut-off level of 4.0 ng/mL for serum PSA. Diagnostic performance of the telomerase activity was analyzed for cut-off level of 100 PC-3 cell equivalent. Therefore, after exclusion of three cases with clinically indefinite diagnosis, we had nine cases with clinically manifested



**Fig. 1.** Analysis of telomerase activity in urine of prostate cancer patient by real-time PCR. Epithelial pool of exfoliated cells harvested by prostate massage was immunomagnetically enriched and isolated from urine of patient with prostate cancer (Gleason score 7; T1cNxMx). Amplification curves (A, family of thin lines) from serially diluted protein extracts of 100,000 PC-3 cells served as a *standard curve* (B) for relative quantification of telomerase activity and as a positive control. The lack of the amplification in highly diluted samples (<10 cells), as well as in samples with omitted protein extracts (negative control) reflected the absence of artifacts, including primer dimers formation. In this case, relative telomerase activity automatically calculated by Opticon MJ Research software was equivalent to the activity of ~10,000 PC-3 cells. Note that all samples can be compared with each other in a linear range above the threshold cycle ( $C_t$ , dotted line).

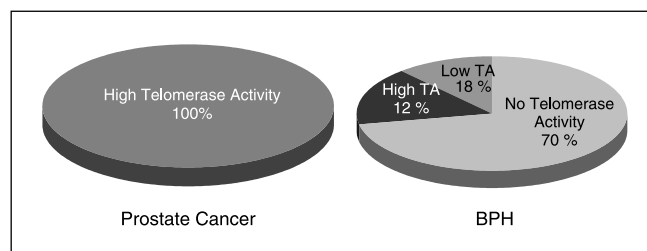
prostate cancer and 44 BPH cases. Because all cancer cases were telomerase positive, a sensitivity of this assay was calculated as 100% (9 of 9). An assay specificity with cut-off level of 100 cell equivalent was 88.6% (39 telomerase-negative cases of 44 BPH patients). Positive predictive value was 64.3% (nine clinically localized prostate cancer among 14 cases with high telomerase activity). A negative predictive value, was calculated as 100% because all telomerase-negative cases were clinically normal (BPH, 39 of 39). Analysis of the PSA levels had shown 88.9% sensitivity for prostate cancer (8 of 9); specificity 76.7% (33 cases with normal PSA of 43 BPH patients); positive predictive value was 50% (among 18 cases with elevated PSA levels, nine were diagnosed with prostate cancer) and negative predictive value was 97% (among 34 cases with normal PSA levels, 33 patients were clinically normal). These data were summarized in Table 2. For telomerase activity, Pearson correlation coefficient was 0.504 ( $P < 0.001$ );  $\kappa = 0.726$  ( $P < 0.001$ ). For serum PSA levels, Pearson correlation coefficient was 0.437 ( $P < 0.001$ );  $\kappa = 0.47$  ( $P < 0.001$ ). Because all prostate cancer cases in this study had high Gleason scores (range, 6-9) and high levels of telomerase activity, but cellular equivalents of relative telomerase activity were variable, no correlation was detected between the levels of telomerase activity and Gleason score.

## Discussion

Our study had principal differences with similar recently published studies (35, 41, 42). First, we collected post-prostatic massage exfoliated cells prospectively, without prior knowledge of clinicopathologic data. Second, we isolated an epithelial pool of exfoliated cells by immunomagnetic cell sorting to exclude possible contribution of other telomerase-competent cells, such as activated lymphocytes or other proliferating cells that might be present in urine. Because tumor cells may be present in low numbers and because the enzymatic activity of telomerase and the PCR efficiency might be affected by the presence of inhibitors in urine, we combined the standardized sample processing with highly sensitive measurements of telomerase activity by quantitative real-time PCR. In contrast

to previously published studies (35, 41, 42) that have reported telomerase activity in exfoliated cells in prostatic fluid and urine in 90%, 91%, and 58% of prostate cancer patients, respectively, in the present study, all patients with clinically localized prostate cancer have shown relatively high levels of telomerase activity that corresponds to 100% of the assay sensitivity. These data are in agreement with basic knowledge that tumor cells exfoliate early during carcinogenesis, *before* the primary tumor can be detected by standard clinical tests (CAP Today Archive, July 1998). The fact that prostate cancer cells do exfoliate into voided urine was confirmed by early studies (12–14) that showed the presence of viable cancer cells of prostatic origin in post-massage samples. In the present study, higher than previously described rates of the detection of telomerase activity in prostate cancer cases can be explained by the use of the real-time PCR technology compared with conventional TRAP assays in all previous reports. Although TRAP assay (24) is the most common strategy for detecting telomerase activity, for prostate cancer, which is characterized by low tumor cell infiltration, the number of exfoliated cancer cells might be insufficient for detection by conventional TRAP techniques that require at least 250 to 5,000 tumor cells (43). In contrast to the end-point PCR amplification, the real-time quantitative TRAP assay allows comparative analysis of samples with different initial amounts of target sequences, because fluorescence signals are measured at the early phase of exponential amplification when reaction products are not limited and the accumulation of inhibitory PCR products is unlikely to occur (44). In addition, RTQ-TRAP assay has several other advantages, such as the exclusion of the time- and resources-consuming post-PCR steps, reliable measurements of telomerase activity in the linear range down to low number of cell equivalents, no interference of primer-dimer artifacts, and relatively high throughput.

**Potential clinical significance of quantitative analysis of telomerase activity.** Whereas the quantitative analysis of telomerase activity has shown 100% sensitivity for prostate cancer, its positive predictive value was estimated at only 64.3% (Table 2), due to the fact that we have detected relatively high levels of telomerase activity (comparable with those of prostate cancer patients) in about 12% of BPH patients. Although these findings obviously conflict with standard clinicopathologic data, they are in agreement with the growing number of reports that the presence of telomerase activity in the cytologically benign lesions may be indicative of the existence of hidden cancer or premalignant disease elsewhere in the gland (28, 34,



**Fig. 2.** Differential expression of telomerase activity in prostate cancer and BPH patients. All patients with clinically localized prostate cancer (*left*) had shown relatively high levels of telomerase activity (100% of the assay sensitivity). The cohort of patients with BPH (*right*, no cancer was detected by standard clinical tests) had shown different levels of telomerase activity: about 70% of cases were telomerase negative, 18% demonstrated low levels, and 12% relatively high telomerase activity, comparable with prostate cancer levels.

**Table 2.** Comparison of the diagnostic performances of telomerase activity and PSA

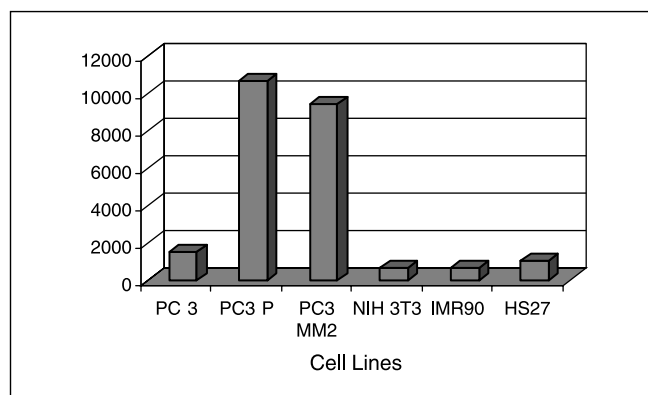
	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Pearson correlation
Telomerase activity	100 (9 of 9)	88.6 (39 of 44)	64.3 (9 of 14)	100 (39 of 39)	0.504 ( $P < 0.001$ )
PSA	88.9 (8 of 9)	76.7 (33 of 43)	50 (9 of 18)	97 (33 of 34)	0.437 ( $P < 0.001$ )

35, 38, 40, 45). Previous studies (37, 38, 40) analyzed telomerase activity by conventional TRAP assay in 10, 16, and 46 surgically removed tissues from BPH patients, respectively, and in urine samples from 30 BPH patients in another study (35). In the present study, telomerase activity was analyzed in a double-blind manner in a cohort of 44 patients with independently obtained clinicopathologic data. Because we analyzed a pool of exfoliated cells that presumably represents all regions of the prostate more objectively than a single set of TRUS-guided biopsies, the positive predictive value of telomerase activity should not be expected to show a good agreement with standard clinical tests. In support of this suggestion, a recent study showed that 10% to 15% of patients diagnosed with BPH by standard clinical tests had T1a and T1b carcinomas (40). It was reported that a second set of biopsies revealed prostate cancer in about 19% of patients with initially negative needle biopsy (8, 10). In addition, a 7-year follow-up study had shown that 11% of patients with negative prostate biopsy were subsequently diagnosed with prostate cancer (45). In a recent study, telomerase activity in prostatic fluid was detected by standard TRAP assay in 13% of BPH patients (35). The authors concluded that it represented false-positive results due to the presence of the inflammatory disease in all cases, with detectable foci of lymphocytes concentration. Although we agree that activated lymphocytes express some levels of telomerase activity, such a contribution should not affect our measurements because we used the immunomagnetic separation of the epithelial pool of exfoliated cells. Because several studies have shown the lack of telomerase activity in pure normal samples, without histologic evidence of prostate tumor anywhere within the gland (28, 42), we suggest that 70% of telomerase-negative measurements in our BPH cases reflect the absence of malignancy, which potentially may be an argument for the exclusion of a large population of patients from further harmful diagnostic procedures. One patient with high levels of telomerase activity in the present study, who was previously misdiagnosed with BPH and inflammation, eventually has revealed the prostate cancer with the Gleason score 7. Therefore, although the clinical significance of positive and negative readings of telomerase activity in BPH cases has to be validated during the long-term follow-up with repetitive quantitative measurements of telomerase activity and repetitive accurate clinical tests, current data suggest that high levels of telomerase activity may be a serious argument to suspect prostate cancer even if it is not confirmed by the first set of standard clinical tests.

It has been shown previously that poorly differentiated cancers with higher Gleason scores are generally associated with the higher rate of telomerase detection and stronger telomerase activity (27–29). The measured levels of telomerase activity may potentially predict the clinical outcome

because high telomerase levels usually correlate with poor prognosis and higher aggressiveness (30). It is reasonable to assume that the telomerase activity is proportional to the number of cancer cells in the sample and to their malignant potential. Our recent data have shown that the levels of telomerase activity correlate with invasive potential of prostate cancer cells. Thus, highly invasive cell lines such as PC3-P and PC3-MM2 had dramatically increased telomerase activity compared with the unmodified and less aggressive prostate cancer cell line, PC-3 (Fig. 3, 47). These results provide a basis for potential application of the quantitative analysis of telomerase activity for discrimination between indolent and aggressive prostate carcinomas. In addition, the quantitative analysis of telomerase activity in urine represents no potential risks and requires no additional costs for sampling; therefore, it can be safely used repeatedly.

Because prostate fluids and urine share the common anatomic duct, the urethra, there is a possibility of the diagnostic bias in case if prostate cancer coincides with other urological malignancies. For example, it was reported that about 3.2% to 3.4% of prostate cancer cases coincide with bladder cancer and that 25% to 70% of bladder cancer cases coincide with prostate cancer (reviewed in ref. 48). Theoretically, cancer cells of other urological malignancies may contribute to the measured levels of telomerase activity, but any potential decrease in the assay's specificity is expected to be minor, due to the significant prevalence of prostate cancer compared with other urological cancers. In the present study, the possibility of coincidental presence of prostate cancer with the high-grade bladder cancer was largely eliminated by



**Fig. 3.** Comparative analysis of telomerase activity in cells with different invasive potential. Highest levels of activity were detected in modified cells with high invasiveness (PC3 P and PC3 MM2) compared with prostate cancer PC-3 cells and several normal cultured cells (NIH 3T3, IMR 90, and HS27). Same protein concentration from different cell lines was analyzed by real-time PCR using serial dilutions of a known number of PC-3 cells as a standard.

monitoring all the samples with the standard urine cytology. In our opinion, early detection of cancer cells of any origin in urine is beneficial for the patient because it will indicate the need for further diagnostic tests focused specifically on urological malignancies.

In conclusion, our data indicate that high levels of telomerase activity in immunomagnetically separated epithelial cells from post-DRE urine specimens strongly suggest the presence of prostate cancer; in contrast, the absence of telomerase activity may be indicative of the lack of prostate

cancer. We also suggest that differential levels of telomerase activity potentially may be used for selection of nonpalpable and potentially aggressive carcinomas that currently have no clinical manifestation. Clinical significance of low levels of telomerase activity in BPH patients needs to be further investigated. It might reflect the presence of indolent noninvasive prostate cancer or early stage of the disease; however, further studies and long-term clinical follow up are necessary to correlate the quantitative analysis of telomerase activity with clinicopathologic data.

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## **Highly sensitive revealing of PCR products with capillary electrophoresis based on single photon detection**

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## Abstract

Post PCR fragment analysis was conducted using our novel DNA sequencing instrument in order to substantially enhance the detection of DNA species. Telomerase Repeat Amplification Protocol assay was used as a model for real time PCR-based amplification and detection of nucleic biomarkers. Using TRAPeze XL kit, telomerase-extended DNA fragments were obtained in extracts of serial 10-fold dilutions of telomerase-positive cells, then amplified and detected in real-time PCR after 40 cycles. Subsequently, characteristic 6-base DNA ladder patterns were revealed in the post-PCR samples with capillary electrophoresis (CE). In our CE instrument, fluorescently labeled DNA fragments separate in a single-capillary separation module where they are illuminated by a fiberized Ar-ion laser. The laser-induced fluorescence (LIF) is filtered and detected by the fiberized single photon detector. To assess the sensitivity of our instrument, we performed PCR at fewer cycles (29 and 25), so that the PCR machine could detect amplification only in the most concentrated samples, and then examined samples with CE-LIF. Indeed, PCR has detected amplification product in samples with minimum  $10^4$  cells at 29 cycles and over  $10^5$  cells at 25 cycles. In contrast, CE-LIF fragment analysis has revealed 6-base repeats in samples with as low as  $10^2$  cells after 29 cycles and  $10^3$  cells after 25 cycles. Thus, we have demonstrated 100 to 1000 fold increase in the sensitivity of biomarker detection over real time PCR, making our approach especially suitable for analysis of clinical samples where abundant PCR inhibitors often cause false-negative results.

**Keywords:** Real-time PCR; Capillary electrophoresis; Single photon detection; Laser-induced fluorescence; Telomerase activity; TRAP

## 1. Introduction

Utmost sensitivity and verifiability in detecting molecular markers are critical but still limiting factors in advancing molecular diagnostics of cancer to point-of-care. There is an urgent need to develop highly sensitive yet cost-effective assays for screening and monitoring biomarkers of the most common malignancies. PCR-based methods can detect markers in much fewer cells than conventional histological or cytological examination. Moreover, methods based on real time PCR exhibit highest sensitivity and range, along with a degree of quantization, which makes real time PCR the most promising basis for the development of molecular methods of cancer detection (e.g., de Kok *et al.*, 2000). The diagnostic value of a particular real time PCR method depends on the fluorescent reporter chemistry being used. Non-specific reporters, such as SYBR Green, which binds to any double-stranded DNA, provide the highest degree of sensitivity. However, SYBR Green can bind equally to primer-dimers and non-specific products as well, providing high incidence of non-specific amplification and false-positive results, so careful validation of outcome is necessary. Use of specific probes, e.g., Molecular Beacons, Scorpions, Amplifluors, etc., ensures higher specificity, but it comes at the expense of sensitivity and thus produces a high rate of false-negative results, which also makes validation necessary.

Sensitivity and validation become even more important when working with real clinical samples. Although several commercial PCR-based telomerase assays already claim single cell (and even single copy) sensitivity, the claims are based on experiments conducted with clean tumor cell lines and pure PCR reagents. In such conditions PCR efficiency is very high (1.8-1.9), yet highly diluted samples become detectable only at very late PCR cycles (40-45). In clinical conditions, however, low levels of input target genetic material are amplified in the presence of various inhibitors of the Taq polymerase,

as well as nucleases, proteases, or other suppressors of biomarkers coming from clinical specimens. This is why efficiency of the PCR amplification in clinical samples usually is much lower than in ideal test samples. For example, several studies evaluating standard assays for telomerase activity showed that the number of cells required for tumor detection varied from 250 to 5000 cells (Muller *et al.*, 1998; Wu *et al.*, 2000b; Isuguri *et al.*, 2002). Obviously, if highly diluted targets in pure conditions are detectable only at very late PCR cycles, clinical samples containing inhibitors and a low number of the target cells may well become undetectable at all. Therefore, a significant (2-3 orders of magnitude) increase in the sensitivity, along with a simple method of validation of PCR products, is needed for PCR to become a reliable tool in clinical practice.

The above consideration motivated us to develop a new concept for a highly accurate and sensitive data acquisition system for the complex quantitative analysis of molecular markers of cancer. It will combine real-time PCR in nano-liter volumes with capillary electrophoresis (CE) using single photon registration of laser-induced fluorescence (LIF) into an integrated data acquisition system operating under a common computer control. The main factor limiting sensitivity of real-time PCR is high background fluorescence associated with probes that are not bound to the PCR product. This background creates high noise, limiting the sensitivity of the system. CE separation eliminates the background fluorescence and allows concentration of the PCR product due to the sample stacking during the electrokinetic injection. Therefore, we expected that the proposed system would have sensitivity several orders of magnitude higher than that of real-time PCR. As a concept-proving step in this direction, we conducted post-PCR fragment analysis with our novel CE sequencing instrument in order to assess the degree to which it enhances the detection of molecular biomarkers. The analysis of *telomerase* was used as a very important and relevant model.

Of about a hundred of discovered cancer-related molecular markers, telomerase has been considered to be the most promising one. Functional telomerase is present in about 90% of human cancers of all tissue types, but, in contrast, it is generally absent from most benign tumors and normal somatic (except germ line and stem) cells (Kim *et al.*, 1994; Shay & Wright, 1996; Dhaene *et al.*, 2000). The detection of telomerase activity has the highest combination of sensitivity (60-90%) and clinical specificity (94-100%) when compared to other screening methods, including cytology and of biochemistry (Hess & Highsmith, 2002; Hiyama & Hiyama, 2002; Isurugi *et al.*, 2002). In many cancers, constant telomerase activity is necessary for tumor development and it appears early in pathogenesis, sometimes in pre-neoplasia state. Therefore, the activity of telomerase has been proven as the most accurate marker for cancer detection, staging and prognosis. (Dhaene *et al.*, 2000; Tatsumoto *et al.*, 2000; Kobayashi *et al.*, 2001; Marchetti *et al.*, 2002).

The ends of chromosomes consist of thousands of double-stranded (ds) TTAGGG repeats called *telomeres* that have several functions, including the protection of chromosomes from degradation and genetic instability (Blackburn, 1991). In normal somatic cells, telomere length is progressively shortened with each cell division, eventually leading to cell death. In contrast, unlimited proliferation of most immortal and cancer cells is highly dependent on the activity of telomerase, which compensates for replicate telomere losses by elongating existing telomere with TTAGGG repeats, using its own RNA component as a template (Ibid.).

Currently, the standard methods for the detection of telomerase activity are based on the telomeric repeat amplification protocol (TRAP) (Kim *et al.*, 1994). They employ the ability of telomerase to recognize and elongate *in vitro* an artificial oligonucleotide substrate, TS, and then use PCR to amplify the extended DNA products. A significant improvement of the TRAP assay is the real-time

quantitative TRAP assay (RTQ-TRAP) (Hou *et al.*, 2001), which combines the conventional TRAP method and a real-time PCR based on SYBR Green. It generates fluorescence signals proportional to the amount of ds amplified product, which is then kinetically measured within a real-time PCR machine. More specific telomerase detection was demonstrated with HEX-labeled TS primer (Atha *et al.*, 2003) as well as using TRAPeze XL kit that employs Amplifluor primers (Elmore *et al.*, 2002). Utilization of labeled primers has the added advantage of enabling fast and simple verification and quantitation of telomerase with CE-LIF (Hess *et al.*, 2004; Jakupciak *et al.*, 2004)

In the present study, we have demonstrated for the first time the use of the TRAPeze XL kit for both real time PCR amplification and CE-LIF detection of fluorescently labeled telomeric repeats. Using a post-PCR fragment analysis of the telomerase extension product in our DNA sequencing instrument, we have demonstrated a 100- to 1000-fold increase in the sensitivity of telomerase detection compared to the sensitivity of the real time PCR method. Preliminary results of this work were reported elsewhere (Kabotyanski *et al.*, 2005).

## 2. Materials and methods

### *DNA sequencer*

For CE separation of the PCR products, we used a single photon detection-based DNA sequencer (Fig. 1 A) that was developed in our group (Alaverdian *et al.*, 2002). DNA samples undergo separation in a single-capillary separation module comprised of a miniature high voltage supply (up to 15kV) with a built-in voltmeter and amperemeter, a polymer replacement system, a temperature control system ( $25-70\text{ }^{\circ}\text{C} \pm 0.01^{\circ}\text{C}$ ), a tube-changer carousel for DNA samples and running buffer, and a precision optical system. When the fluorescently labeled DNA fragments pass the optical system (Fig. 1 B), they are illuminated by a fiberized Ar-ion laser (488 and 514nm, 20mW, Uniphase, CA, USA). The LIF is filtered and detected by the fiberized single photon detector (APD, SPCM-AQ4C, Perkin Elmer, CA, USA). A photon counting circuit board based on field programmed gate array (FPGA) technology is used to count, integrate, and transfer the data to a computer.

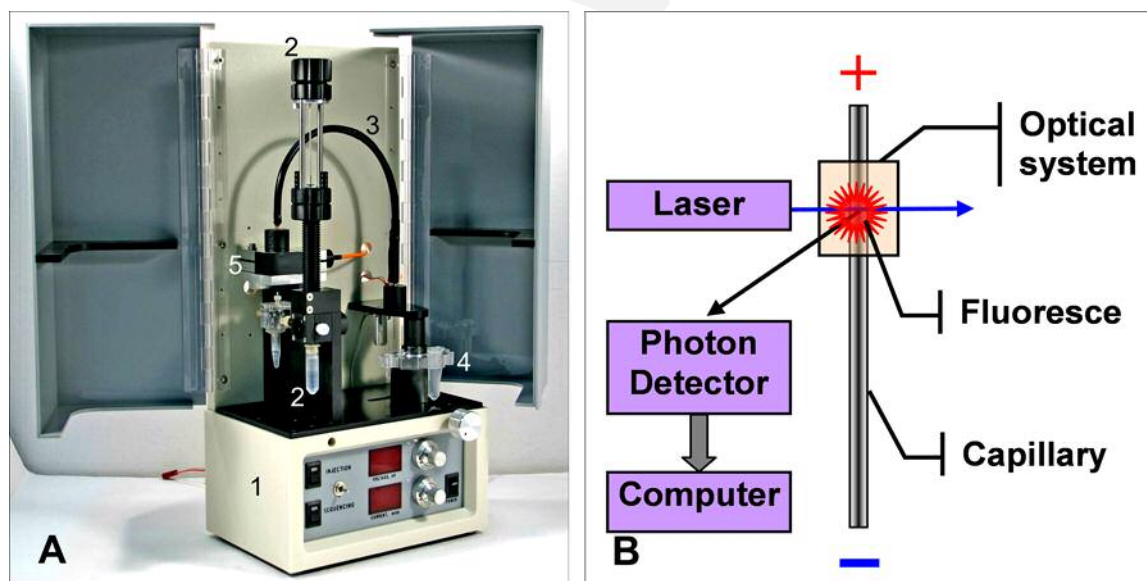


Fig.1. Single photon detection-based DNA sequencer. A: general view. 1 - high voltage supply with a built-in voltmeter and amperemeter; 2 - polymer replacement system; 3 - temperature control system; 4 - tube-changer carousel; 5 – fiberized precision optical system. B: schematic principle of the instrument.

### **PCR assays**

**Protein extracts from telomerase-positive cells.** One source of telomerase was the control cell pellet ( $10^6$  telomerase-positive cells) provided with the TRAPeze XL Telomerase Detection Kit (Chemicon, CA). Alternatively, PC-3 cells were provided by the Cell Culture/Hybridoma facility (SUNY Stony Brook, NY).  $10^6$  cells were pelleted, washed with ice-cold PBS, and repelleted. Cells pellet were resuspended in ice-cold CHAPS lysis buffer containing 100 U/ml of SUPERase (Ambion) and incubated on ice for 30 min. Lysates were then centrifuged (16000 g, 20 min, 4° C). The supernatant fluid was removed, aliquoted, snap frozen and stored at -80° C. Stock aliquots were serially diluted 1:10 in CHAPS lysis buffer ( $10^6$  to 10 cells per 20  $\mu$ l); 2  $\mu$ l of each aliquot were further used for RTQ-TRAP (final dilutions were  $10^5$  to 1 cells).

**RTQ-TRAP** was performed in a 96-well plate format using DNA Engine Opticon (MJ Research, MA). QuantiTect SYBR Green PCR Kit (Qiagen, CA) and TRAPeze XL kit were used according to manufacturers' instructions with some modifications.

**Telomerase extension:** Reaction mixtures of either kit (25  $\mu$ l / well) were first incubated at 25° C for 30 min to allow the telomerase in the extract to elongate the TS primer. The products of this extension reaction, DNA molecules with various numbers of telomeric repeats in 6 base increments, served as templates for the following PCR. In some experiments, TSR8 control template DNA (Chemicon) in final concentrations of 0.02, 0.002, and 0.0002 amoles/  $\mu$ l was used as positive control. Reaction mixture without cell extract was used as a negative control. All samples were examined in duplicates.



**QuantiTect Kit:** Primers (telomerase substrate and forward, **TS**: 5'-AATCCGTCGAGCAGAGTT-3'; reverse, **ACX**: 5'-GCGCGGCTTACCCTTACCCTTACCCTAACC-3') (Invitrogen, CA) were added to reaction mix prior extension at 0.2  $\mu$ M. *Cycling*: 95° C for 15 min (hot start), followed by 40-cycle amplification (denaturation at 95° C for 30 sec; annealing at 55° C for 30 sec; extension for 60 sec and plate reading for 10 sec at 72° C), and 4° C hold. In some experiments the melting curve was read from 65 to 95° C.

**TRAPeZe XL Kit:** Primers: The fluorometric detection is based on the Amplifluor primers, whose working is well explained in the manual ([www.chemicon.com/webfiles/PDF/S7707.pdf](http://www.chemicon.com/webfiles/PDF/S7707.pdf)) and in Elmore *et al.* (2002). The reaction mixture already contains **TS** forward and fluorescein-labeled **Amplifluor RP** reverse primers that generate a ladder of green-fluorescing products. It also contains a sulforhodamine-labeled Amplifluor K2 reverse primer, which is involved in the semi-competitive simultaneous amplification of the internal control template TSK2 generating a red-fluorescent 56 bp product. Wild type (FastStart by Roche, IN) or Titanium (Clontech, CA) Taq DNA Polymerases were added to the reaction mix at 0.5  $\mu$ l (equivalent of ~2.5 wild type Units) also prior extension. *Cycling*: 95° C for 1 min (hot start), followed by 40-cycle amplification (denaturation at 94° C for 30 sec; annealing at 55° C for 30 sec; extension for 60 sec and plate reading for 5 sec at 68° C), 3 min incubation at 68° C, and 4° C hold. In some experiments the melting curve was read from 65 to 95° C after final 3 min incubation at 55° C.

## CE

Polyimide coated fused silica capillary tubing (TSP050375, Polymicro, AZ), 50  $\mu$ m ID, was used. LIF was detected at 50 cm, where 1 cm of coating was removed by heat.

***CE in non-denaturing gel.*** PCR products labeled by SYBR Green I with QuantiTect kit were separated in non-denaturing Genescan Polymer (GSP) (Applied Biosystems, CA), 5% in ABI buffer. Post-PCR samples were diluted 1:5 in ultra pure H<sub>2</sub>O, then electrokinetically injected (25 seconds, 10 kV) and separated at 8 kV at 25°C.

***CE in denaturing gel.*** Fluorescein-labeled PCR products obtained with TRAPeZe XL kit were separated in denaturing POP-7 gel (Applied Biosystems). Post-PCR samples were usually used without clean-up, but in some experiments they were first de-salted with Performa spin-columns (Edge BioSystems, MD) by spinning 2 min at 750 g. Samples for CE were prepared in two ways. One was mixing 1 µl of PCR product with 9 µl Hi-Di formamide (Applied Biosystems), heating the mixture for 3 minutes at 94° C and flash-chilling on ice. Another was mixing 2 µl of PCR product with 2 µl Hi-Di formamide, heating the mixture for 3 minutes at 94°C and flash-chilling on ice, then mixing it with 16 µl of ice-cold ultra pure H<sub>2</sub>O. Samples were usually electrokinetically injected at 5 kV for 40 sec, and separated at 8 kV at 50° C. In some instances, 0.5 µl of Internal Lane Standard 600 (ILS) (Promega, CA) was added to a sample for size scaling.

### ***Data processing and analysis***

Data were collected and analyzed using our software, which includes separate modules for run data recording (MONITOR), processing, visualization, and editing (BASE). The MONITOR accepts the sampled sequencing data transferred via the Parallel Port, performs its real time visualization and recording to a 'raw' on the hard drive. The BASE produces fully processed *PHRED-compatible* data files as its output and provides a visual interface, which allows viewing, manual processing, editing and printing of both 'raw' and processed data.

### 3. Results

#### *SYBR Green-labeled PCR products*

**PCR.** Using QuantiTect kit, we obtained a series of amplification curves corresponding to serial 10-fold dilutions of extracts of telomerase positive cells ( $10^5$  to 0.1 cell) (Fig. 2, A and B). The data were similar to those obtained previously in our group (Botchkina *et al.*, 2005) or by others (Wege *et al.*, 2003). These experiments also demonstrated that the assay had a limited linearity: there was some inhibition at high concentrations ( $10^5$  cells - left-most curve, red), while at lower than 100 cell concentrations there was either no amplification (1 and 0.1 cell) or the kinetics were such that they suggested a false positive result (10 cells - right-most curve, blue). These results agree with previous reports that the TRAP-based assays have a limited linearity of (Wu *et al.*, 2000b), and that telomerase-positive cell lines may harbor only 1 telomerase molecule per 2 to 6 cells (Meid *et al.*, 2001). Accordingly, in the following experiments we used only 5 dilutions – from  $10^5$  to 10.

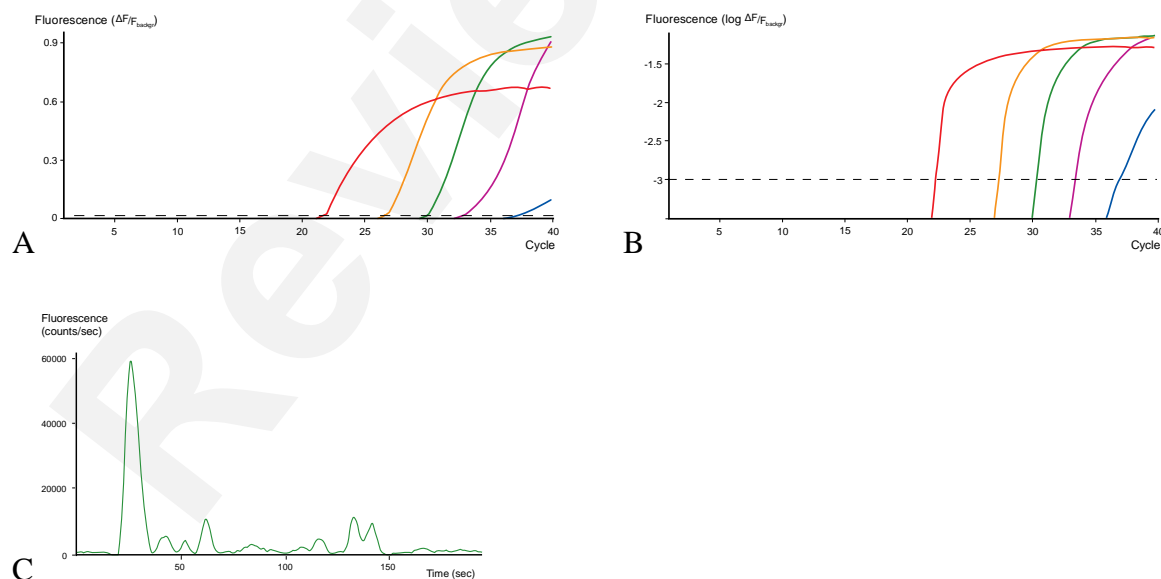


Fig. 2. Real-time PCR with QuantiTect kit followed by CE. *A*: SYBR Green fluorescence values were obtained from wells with corresponding serial dilutions of cell extracts (from left:  $10^5$  to 0.1 cells) and plotted against cycle numbers. 1 and 0.1 cell and negative control had 0 values (not shown here). Dashed line – threshold at which Ct values were determined. *B*: same as *A*, in semi-log scale; right-most curve for 10 cells (blue) reveals distinct kinetics. *C*: electropherogram, baseline removed, of  $10^5$  cells sample in GSP gel; earliest and largest peak appears to represent primers.

**CE.** After PCR, solutions containing amplified telomeric products were subject to CE-LIF fragment analysis. CE in a denaturing gel (POP 7, ABI) predictably gave negative results (not shown), because SYBR Green is non-covalently bonded to dsDNA. Fluorescent material was detected only with non-denaturing GSP gel. Only at the highest concentrations ( $10^5$  and  $10^4$  cells) was fluorescence significantly different from that of negative controls and appeared as DNA repeats ladder (Fig. 2, C). Thus, CE-LIF of material obtained with SYBR-based chemistry does not exhibit any benefits over PCR itself. We next used TRAPeZe XL kit that employed covalently-labeled primers.

### ***Covalently-labeled PCR products***

**Optimization.** First, we optimized real-time PCR with this kit, since it was not specifically designed for real-time, and in the only work known to us on real-time PCR with TRAPeZe XL, optimization was not reported (Elmore *et al.*, 2002). One variable is DNA polymerase, so we compared widely used wild type and Titanium Taq Polymerases. The latter is a mutant with the loss of the 5'-exonuclease activity and with increased solubility. Using cycling parameters for QuantiTect kit, we have found that Titanium Taq resulted in higher PCR efficiency and/or increased sensitivity, as manifested by steeper slopes of the amplification curves and lower Ct values (data not shown here).

Other variables include temperature and timing of PCR cycles. We set durations of each step of a cycle according to TRAPeZe XL manual. Temperatures, however, appeared to be critical for real-time

detection of fluorescence. Unlike with SYBR Green, where maximal saturation signal exceeds background signal by ~100%, with Amplifluor chemistry maximal signal exceeded background by ~40% at plate read temperature of 72° C. Measuring melting curves revealed that as temperature increased, fluorescence changed in reverse directions, depending on the concentration of PCR product. In negative control or low abundance samples, background fluorescence was low at 65° C and gradually increased toward 80 – 85 °C, whereas in wells with abundant amplicons the fluorescence was maximal at 65 °C and then decreased to background level at 80 – 85 °C (Fig. 3, A). For practical purposes, this means that the signal to background ratio is better at lower temperatures. Therefore, we had decreased plate reading temperature to 68° C. In order to keep cycling fast and simple, we also decreased the temperature of the preceding extension step to 68° C as it was still within optimum range of Titanium Taq. As a result, signal to background ratio had increased to 0.6. The results described below were obtained with the optimized PCR parameters detailed in Methods.

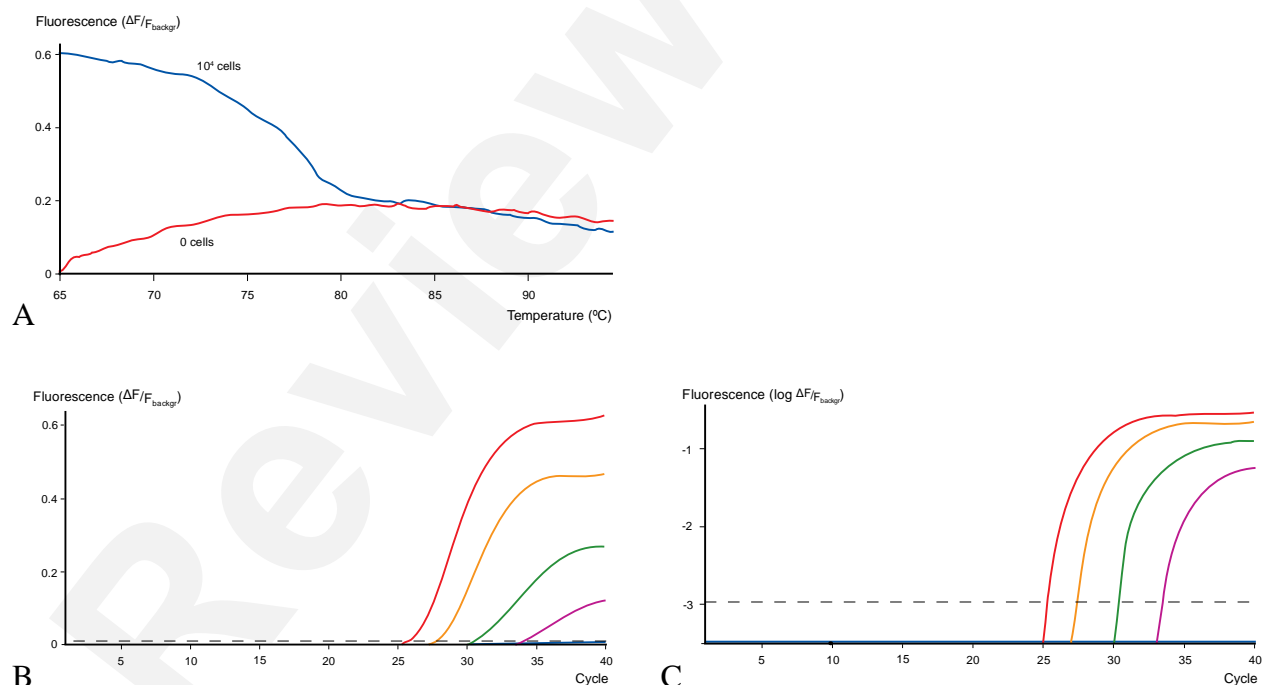


Fig.3. Real-time PCR amplification with TRAPEze XL kit. **A:** melting curves (relative intensities) recorded after PCR from wells with negative control (lower, red) and  $10^4$  cells extract (upper, blue); their dynamics are reverse and differences diminish with the increase of temperature. **B:** amplification curves obtained from wells with extracts of serial cell dilutions (from left:  $10^5$  to  $10^2$  cells). 10 cell

extract exhibited no amplification – bottom line, blue. Dashed line – threshold at which Ct values are determined. *C*: same as *B*, in semi-log scale.

**PCR.** Our results confirmed that TRAPeZe XL can be used in the real-time PCR procedure (Fig 3, B and C). The sensitivity of this kit was very similar to the sensitivity of the QuantiTect kit, based on Ct values for same dilutions. The important difference was that for 10 cells extracts, there was no amplification detected, which additionally indicated that in QuantiTect kit, amplification in 10 cells samples was false-positive. Because of very high specificity of this assay, lack of amplification in 10 cells extracts appeared to be true negative. However, because 10 cells may contain 1 to 5 active telomerases (Meid *et al.*, 2001), this result needed further validation with CE-LIF.

Ct values for  $10^5$  cells were 25-26 cycles (median 25); for  $10^4$  cells – 27-29 (median 28); for  $10^3$  cells – 29-31 (median 30); and for  $10^2$  cells – 32-36 (median 34). Note that the slopes of amplification at semi-log linear phase (Fig 3, C) are not as steep as with QuantiTect (Fig 2, B). This indicates somewhat slower kinetics of PCR with TRAPeZe, which in part could be explained by a competition from internal control templates for the TS primer. In addition, unlike with the QuantiTect kit, fluorescence at the saturation phase is proportional to initial concentration. This could be explained by the original undisclosed design of TRAPeZe XL intended for end point PCR quantitation.

**CE.** Next, samples were subjected to CE-LIF fragment analysis. Since our instrument detects in four color bands ( $546 \pm 5$  nm,  $560 \pm 5$  nm,  $590 \pm 5$  nm, and  $610 \pm 5$  nm wavelengths with barrier filter  $540 \pm 5$  nm), we used color deconvolution to separate red labeled control DNA amplicons and green labeled telomerase extension repeats. For the red color, a transfer matrix was obtained from electropherograms of negative control samples (Fig. 4A). The matrix was taken from the peak corresponding to red

fluorescing sulforhodamine-labeled internal control amplicons. For the green color, we used  $10^3$  cells samples; the matrix was taken from one of the peaks corresponding to green fluorescing telomerase-extended DNA. Co-injection with the ILS calibration ladder proved that the red peak corresponded to 56 bases, whereas the green peaks were 6 bases apart and corresponded to 55, 61, 67, etc. bases (Fig. 4B). This matched the sizes of the control template and telomere repeats ladder expected from the kit manual, except for the 55 base peak not mentioned there.

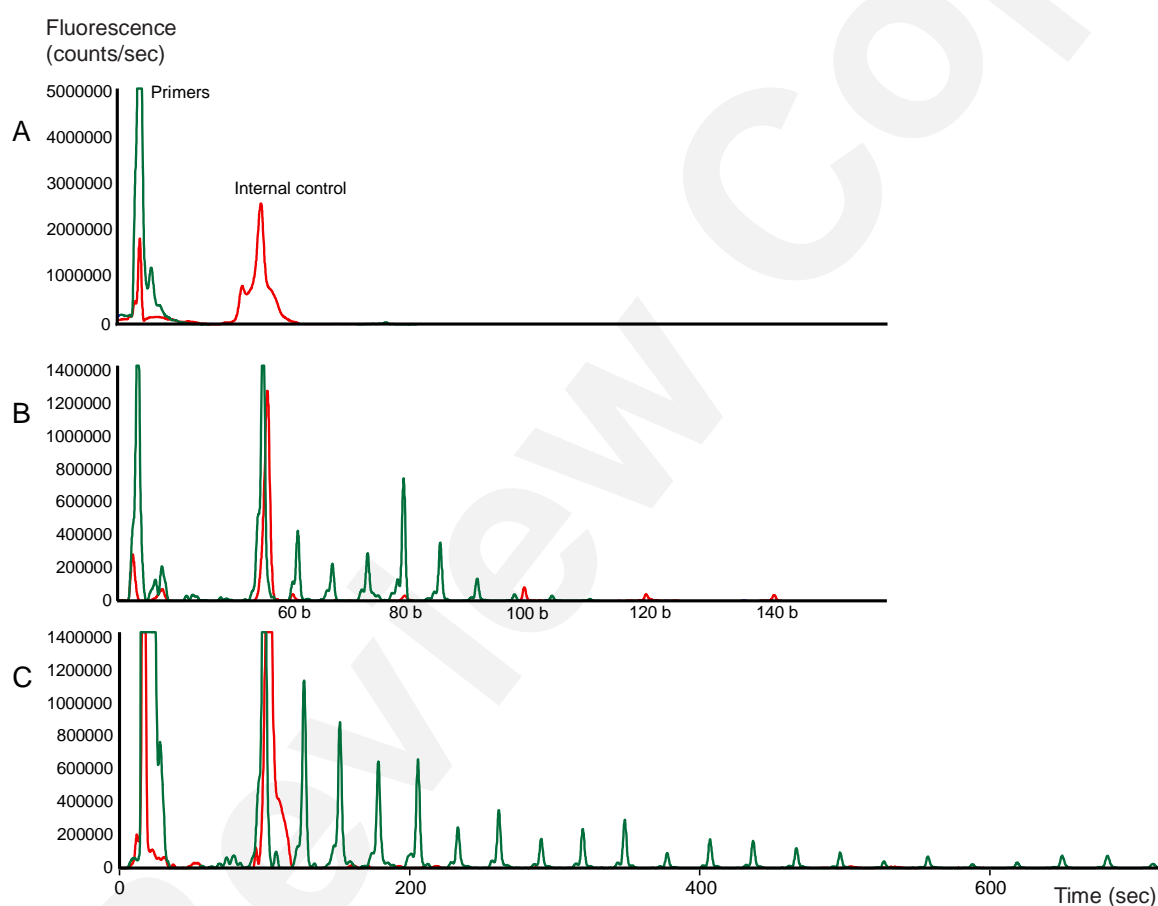


Fig.4. Color-deconvoluted, baseline removed, electropherograms of samples containing negative control (A),  $10^2$  cells + ILS (B), and  $10^2$  cells from optimized injection experiment (C) after real-time PCR with TRAPeze XL kit. Traces of red-fluorescing material are depicted here and below in red color, green fluorescing – in green. Small red peaks in B are ILS' 60, 80, etc., base marks, as indicated.

**Optimization.** We aimed not to use post PCR clean up for simplicity of the protocol and obtained good results because only specific products are labeled with fluorophores due to the chemistry of the kit.

When Performa spin columns were used for sample desalting, we observed a significant increase (two to three times) in signal amplitude on electropherograms (not shown here).

Initially samples were prepared for CE as widely done in the field: diluting post PCR solution in formamide 1:10 and denaturing before electrokinetic injection. Results were satisfactory (Fig. 4, A, B). However, since formamide is hundreds of times more ionized than ultra pure H<sub>2</sub>O, we modified sample preparation so that injection medium was less ionized and thus favored electrophoresis of DNA molecules. We first denatured and stabilized DNA in a smaller amount of formamide (1:1), and then further diluted it 1:5 in ultra pure H<sub>2</sub>O (see Methods). This protocol further improved electrokinetic injection (Fig. 4, C). Peak amplitude increased by  $53 \pm 18 \%$  and more telomeric repeats peaks could be distinguished (up to 26).

Optimal injection parameters were found to be 5-8 kV and 60-300 seconds. Higher injection voltages (up to 10 kV) and longer injection times (up to 10 minutes) led to the distortion of the peaks pattern at the early (lighter) portion of a pherogram. However, the amplitude of fluorescence of heavier bands continued to increase without much distortion, so these extreme injection parameters could be used to obtain a signal from much diluted samples.

In CE, 10 cells samples exhibited pattern identical to that of negative control, thus proving that negative PCR results obtained for this dilution with TRAPeze XL kit were true. This result underlines the need of validation of real-time PCR results obtained for low abundance samples using such non-specific probes as SYBR Green. It also indicates that one active telomerase probably occurs in over 10 cells. To exclude insufficient sensitivity of our CE-LIF measurements, we assessed it by filling the capillary with nanomolar dilutions of fluorescein (Fluka). We determined that for 50 $\mu$ m ID capillary



and 20 $\mu$ m diameter laser beam, a peak must contain over 100 of labeled molecules in order for it to be detectable by LIF. This is 4-10 orders of magnitude lower than the number of amplicons in post-PCR sample. Thus the sensitivity of our CE-LIF is ample.

***Sensitivity of telomerase detection.*** Notwithstanding the above estimates, the PCR method appeared to be sensitive enough to detect the minimal activity of telomerase in the experimental settings we used. These were model, ideal conditions: proliferating cells grew in homogeneous culture, they were collected and extracted protein fraction in such a way that excluded ingress of factors that may retard PCR. Indeed, our data suggest that the efficiency of PCR was close to the theoretical maximum of 2. In real clinical or forensic practice, however, samples are collected in biopsy, washout, blood, etc. They contain many non-proliferating cells, in which telomerase is suppressed by endogenous factors (Akane *et al.*, 1994; Wu *et al.*, 2000a). In addition, specimens contain extracellular inhibitors, such as hemoglobin or mioglobin (Akane *et al.*, 1994). Thus, clinical samples contain a variety of inhibitors, which may impede telomerase activity and decrease the efficiency of RTQ-TRAP to below 1.5. As a result, PCR may become insufficient to amplify the diluted template DNAs during 40-50 cycles to a threshold level necessary for detection. This may lead to false negative results, missed identification of biomarkers, and missed diagnosis. These are the situations in which validation of PCR results with much more sensitive CE-LIF becomes critical.

In order to quantitatively compare sensitivities of PCR and CE-LIF, we modified our experimental model so that it emulated PCR inhibition and false negative results. We could not achieve this by simply further diluting cell concentrations because 10 and less cell dilutions exhibit no telomerase activity. One way to model PCR inhibition would be to mix PC-3 cells with various cell populations and/or hemoglobin. However, this tactic seems to lessen quantification of the results and compatibility

with the preceding experiments. We chose another approach: to reduce the number of PCR cycles. By this, we quantitatively mimic reduction of efficiency (hence sensitivity) of PCR, as if in case of inhibition, and generate known false negative samples for further CE-LIF analysis. At the same time, we controllably change only one parameter, while other experimental conditions remain the same. Our logic is as follows; If, for example, we carry out only 29 cycles of PCR, then, based on the results described above (Fig. 3), we expect PCR to detect telomerase activity only in  $10^5$  and  $10^4$  cell dilutions.  $10^3$  and  $10^2$  cell dilutions are expected to yield negative results because their fluorescence would not yet overcome the threshold of being significantly higher than background. These samples would be known false negatives. Then these samples will be subjected to CE-LIF fragment analysis. If our instrument detects telomeric repeats in  $10^3$  cells sample – then sensitivity of our device would be  $10^4/10^3 = 10$  times higher than that of PCR. If it detects the 6 base repeats ladder in  $10^2$  cell sample – then we could claim  $10^4/10^2 = 100$ -fold increase in sensitivity.

*Reduced PCR.* Thus, we next conducted real-time PCR for 29 cycles. Sample preparation and TRAP-PCR conditions were as indicated above, except Opticon machine was stopped after 29 cycles. Only  $10^5$  cells extract produced fluorescence significantly higher than background (Fig. 5, A, B, red line). In this particular experiment,  $10^4$  cells (brown line) produced just under threshold level of fluorescence. It should have been considered a negative along with the solutions of lower cell concentrations ( $10^3$  – blue,  $10^2$  – green) because their fluorescence is indistinguishable from background fluorescence. Since in 40 cycles PCR the fluorescence of  $10^4$  cells samples crossed the threshold on average at about 28<sup>th</sup> cycle, we considered  $10^4$  cells as sensitivity threshold for 29 cycles PCR. Samples containing  $10^3$  and  $10^2$  cells were considered known false negatives because, even though they yielded negative results with 29 cycles PCR, we knew from 40 cycles PCR and CE-LIF that they did contain telomeric repeats.

In addition to 29 cycles PCR, we conducted real-time PCR at further reduced number of cycles – 25. No amplification was detected in any of the samples (Fig. 5, C). Based on the average from 40 cycles experiments,  $10^5$  cells should be considered a sensitivity threshold, and  $10^4$ ,  $10^3$  and  $10^2$  cells samples as known false negatives for the 25 cycles PCR.

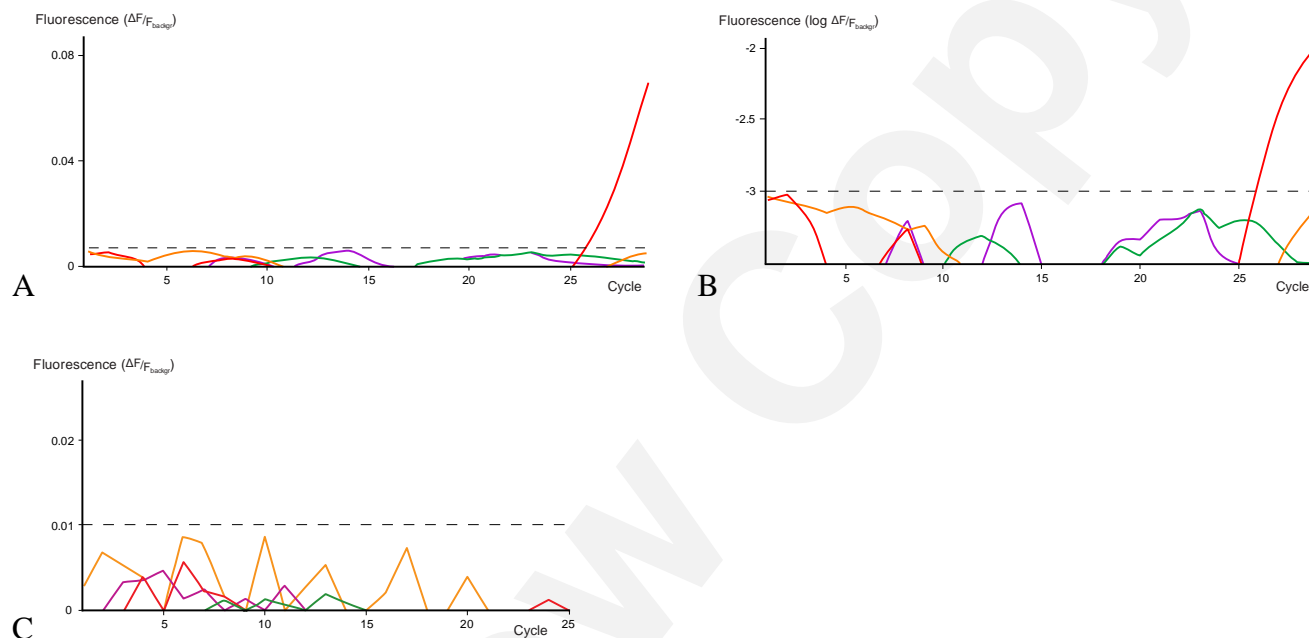
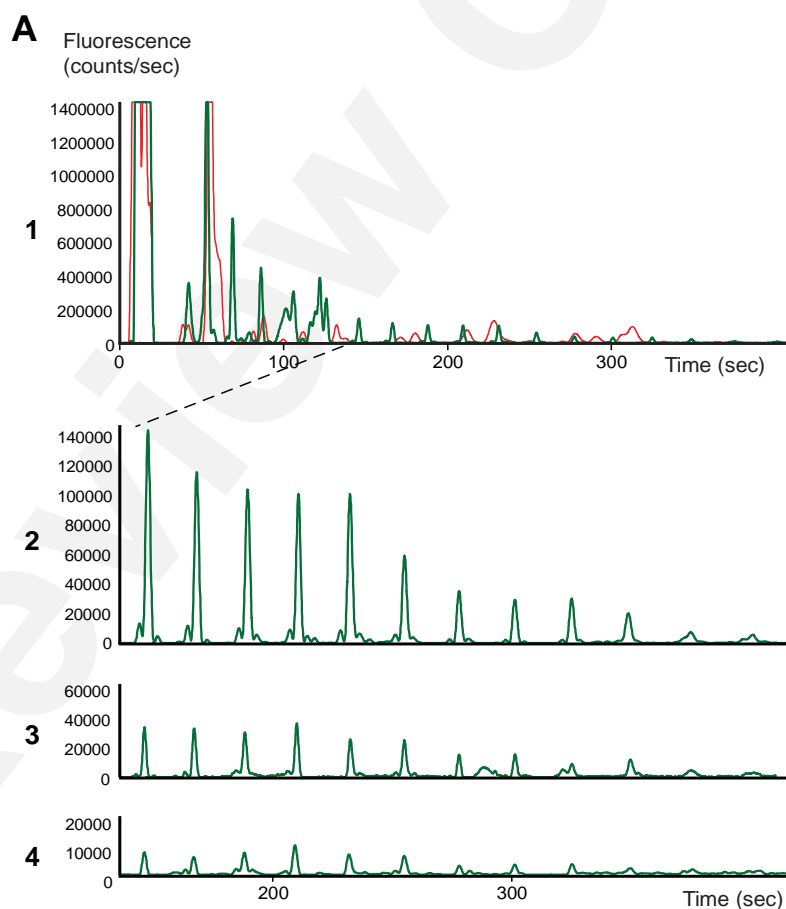


Fig.5. Amplification with short real-time PCR.  $10^5$  cells – red line;  $10^4$  cells – orange;  $10^3$  – green; and  $10^2$  – purple. *A*: 29 cycles, linear scale. *B*: same, semi-log scale. Only  $10^5$  cells sample exhibited detectable amplification during 29 cycles PCR. *C*: 25 cycles, linear scale. Only fluctuations of background fluorescence were detected during 25 cycles PCR. Note that Fluorescence scales in this figure are about 1/10 of those in Figs. 2 and 3 because of limited amplifications.

*CE.* Under CE-LIF examination, we observed that the electropherograms of samples after 29 cycles PCR exhibited much higher fluorescence for primers and much lower for internal control and telomeric repeats than that observed for corresponding cell concentrations after 40 cycles of PCR. This was expected from exponential kinetics of PCR, due to which most of amplified material is produced at the later cycles. In order to compensate for this, we injected samples at 7kV for 320 sec, and ran CE at 10 kV. As a result, the peaks' amplitudes have increased, but this also resulted in increased non-specific

fluorescence of contaminants or debris, especially at lighter fractions (up to 80 bases) that also include peaks of 1<sup>st</sup> to 5<sup>th</sup> repeats (Fig. 6, A1, 10<sup>4</sup> cells). Therefore, we focused on examining and comparing peaks that start from the peak of the 6<sup>th</sup> repeat (Fig. 6, A2) in order to determine whether our instrument can detect telomeric repeats at 10<sup>3</sup> and 10<sup>2</sup> cells concentrations (Fig. 6, A3 and A4). Indeed, as demonstrated in these figures, photon counting-based CE-LIF technology has revealed telomeric repeats at all cell concentrations. In particular, characteristic repeats ladder pattern was detected in samples with 10<sup>2</sup> cells extract (Fig. 6, A4), which is 3 orders of magnitude lower than the lowest concentration detected in this experiment by PCR technology (i.e., 10<sup>5</sup> cells), and 2 orders of magnitude lower than average sensitivity threshold for 29 cycles PCR (i.e., 10<sup>4</sup> cells). As explained above, this means that in this experiment, CE-LIF had 1000- fold higher sensitivity than PCR.



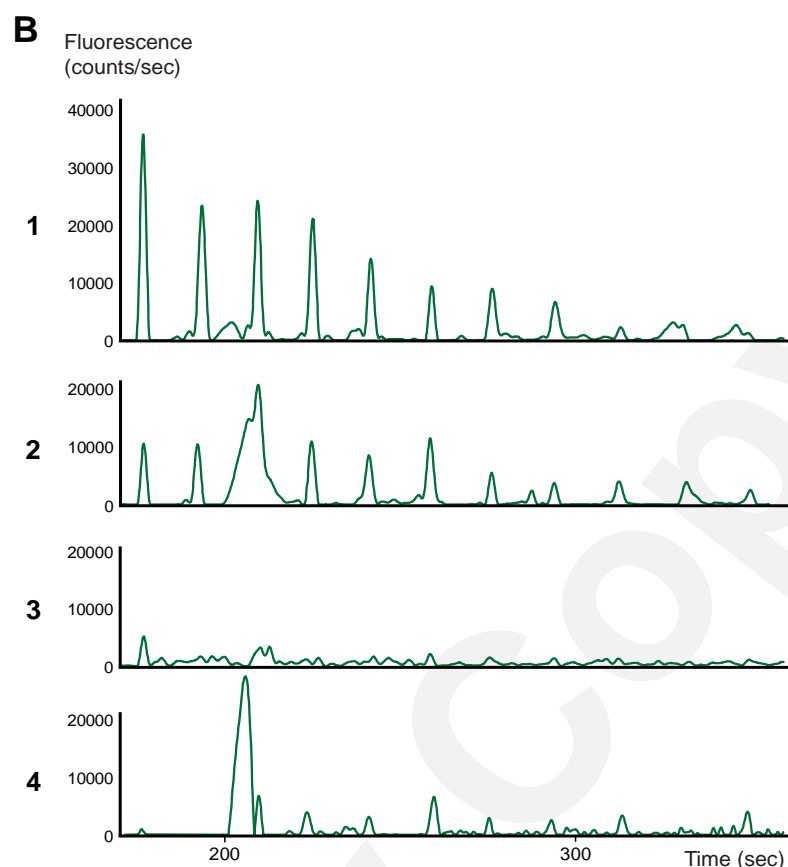


Fig.6. Electropherograms of samples after 29 (A) and 25 (B) cycles of PCR amplification with TRAPeZe XL kit. A: 1 - extract of  $10^4$  cells; 2 - expanded and amplified area of A1, peaks from 6 to 17; 3 and 4 - expanded and amplified area of peaks 6 to 17 from samples with  $10^3$  (3) and  $10^2$  (4) cells extracts. B: expanded and amplified area of peaks 6 to 16. 1 - sample with  $10^5$  cells extract; 2 -  $10^4$  cells; and 3 -  $10^3$  cells; 4 - same sample as in 3, after desalting and 620 s electrokinetic injection. In B2 and B4, peak 8 was co-separated with debris; and in B4, peaks 7 and 15 were not detected. Traces for red-fluorescing material were omitted in panels A2 – B4.

Finally, we analyzed samples that have undergone only 25 cycles of PCR. In the CE-LIF instrument, telomeric repeats were detected in  $10^5$ ,  $10^4$ , and  $10^3$  cells samples (Fig. 6, B1-B3). Desalting  $10^3$  cells samples and prolonging injection to 620 sec further increased the amplitude of telomeric repeat peaks (Fig. 6, B4). Since no amplification was detected by 25 cycles PCR even in  $10^5$  cells sample (Fig. 5), data for the  $10^5$  cells extract was known false negative, and 25 cycles PCR sensitivity threshold was over  $10^5$ . Thus, in this particular experiment sensitivity of CE-LIF also proved to be over two orders of magnitude higher than that of PCR ( $>10^5/10^3 = >10^2$ ). On average, though, CE-LIF technology again exhibited 100 times higher sensitivity than 25 cycle PCR.

#### 4. Discussion and Conclusions

Using 25 and 29 cycle real-time PCR to model sub-efficient PCR in clinical samples, we have demonstrated that post-PCR fragment analysis with our single photon counting-based CE-LIF system yielded over 100 times higher sensitivity than most sensitive and specific real-time PCR. We believe that this ratio can be further improved by at least another order of magnitude. For example, we did not use any special methods for signal analysis and recorded telomeric repeats signal on the background of noise. However, periodicity of telomeric repeats permits improving their signal with, for example, auto-correlation analysis. Moreover, while in RTQ-TRAP we detected integral signal from a ladder of amplicons in a sample, in CE their signal was divided into 10 to 20 peaks separated by 6 bases. Thus, it is reasonable to expect 10 times higher signal from single band/peak amplicons. Signal also can be improved by post-PCR clean-up of DNA, removal of primers, etc. In addition, we can optimize the CE-LIF instrument by using optimal filters with wider band; we can increase photon collection times (thus signal) fourfold by using one filter instead of rotating four. Finally, there is a potential of modifying TRAPeze XL kit to improve its performance in real-time PCR and CE-LIF.

Although telomerase, which is expressed in almost 90% of human cancers, is one of potential clinical molecular markers for the disease, there has not been developed a telomerase assay suitable for clinical testing. While PCR assays are sensitive, they have limited potential for real clinical samples because in the presence of inhibitors, decreased PCR sensitivity leads to false negative results. Here we described the development of a highly sensitive CE-LIF-based assay for validation of telomerase activity. In this study, for the first time, we have demonstrated the use of the TRAPeze XL kit for both real time PCR and CE-LIF; optimized PCR and CE protocols for this kit; and established that photon counting-based CE-LIF has over two orders of magnitude higher sensitivity than real-time PCR with TRAPeze XL.

We compared PCR methods based on different chemistries – intercalating or covalently labeled probes. When combined with SYBR-based chemistry, CE did not offer any benefits over PCR because SYBR dyes are known to significantly retard and distort electrophoresis (Tuma et al., 1999). Moreover, fragments moving through the gel matrix appear to shed non-covalently bonded SYBR Green due to interference with the gel matrix and thus lose fluorescence signal. The advantage of covalently labeled primers is that, when they are extended during PCR, each amplicon molecule has one covalently bonded label. Although the total signal may be smaller, this benefits CE: electroseparation of fragments is more quantitative and accurate than with intercalating dyes, which bind proportionate to dsDNA length and thus cause a significant shift. In addition, ssDNA can be assayed for additional accuracy, and CE can be performed with a variety of advanced denaturing gel matrices. One of a few PCR kits based on this kind of chemistry is the TRAPeZe XL kit, and it is the only one on the market for telomerase detection.

Quality of PCR data obtained with TRAPeZe XL kit was further improved by reading plate at lower temperatures where the difference between signal and background was larger. This phenomenon can be explained by the nature of Amplifluor primers: at low temperatures the hairpin loop is predominantly folded and thus energy transfer quenching within the loop is most efficient; at higher temperatures, a larger proportion of loops may stochastically open / stay open for longer times and thus allow fluorescein to emit. In PCR-extended product, however, hairpin is unfolded in dsDNA and fluoresces, but with the increase of temperature, more dsDNA molecules de-hybridize, and more hairpin structures are released and folded. At 80 – 85 °C, apparently all hairpin structures become single stranded and reach the same dynamic balance between folded and unfolded states as do unextended primers. This is probably why at these temperatures fluorescence of primers and amplicons becomes identical (Fig.

3A). In future experiments, we plan to take melting curve at wider range of temperatures, and to investigate the possibility of further optimization by taking plate readings at much lower temperatures, e.g., 50 – 55 °C.

Post-PCR fragment analysis had also demonstrated that there were no telomeric repeats present in 10 and lower cell extracts. This corresponds to earlier findings that TRAP assays could not reliably detect telomerase activity at concentrations lower than 250 cells (Wu *et al.*, 2000b). Since our CE-LIF instrument has at least 100 fold higher sensitivity than PCR, we can exclude false-negative result due to the low sensitivity of TRAP assay. Because in model conditions that we used, the PCR method, according to manufacturer, has single-molecule resolution, this result suggests that one active telomerase may occur in between 10 to 100 cells. Given that we used telomerase-positive cells from cell culture lines, this suggestion may contradict the postulate that each immortal cell has an active telomerase. There could be a number of reasons. For example, only a percentage (1-10%) of an immortal population is truly immortal cells that keep proliferation and have telomerase activity. Another possibility – only a portion of the telomerase pool is active at a given point of time or cell cycle. In addition, there is a loss of enzyme during extraction of protein fraction for TRAP. All this further emphasizes the need of validation of real-time PCR results obtained for low abundance samples, and it questions usefulness in clinical practice of non-specific probes as SYBR Green.

In conclusion, we believe that the unique sensitivity of our instrument makes it especially suitable for molecular analysis of clinical samples, in which PCR is suppressed, may not yield a detectable signal, and may lead to missed detection and diagnosis of a disease. These are the situations in which ultra sensitive CE analysis of PCR products becomes critical because it enables their verification, elimination of false positive and false negative results, and thus correct interpretation of the data.



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Review Copy

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# Prostate test advances

■ Researchers at Stony Brook are developing an easier and more accurate method of finding malignancies

BY DELTHIA RICKS  
STAFF WRITER

Scientists at Stony Brook University Hospital have cleared the first step in studies to develop a noninvasive test that accurately spots a prostate cancer-specific enzyme, promising a new way to diagnose the disease, the team's lead member said yesterday.

The diagnostic tool relies on an enzyme called telomerase, which is known to be inactive when cancer is not present but flares when a malignancy occurs. In the case of prostate cancer, the enzyme is found in abundance in urine.

Galina Botchkina, who studied 56 Long Island men, was able to accurately diagnose prostate cancer in all nine of those who developed the disease.

"We need to confirm this in a very large population of men," said Botchkina, a molecular bi-



PHOTO BY PATRICK OEHLER

**'It is really important to develop new molecular tools so that we have more accurate diagnosis.'**

— Stony Brook researcher Galina Botchkina

ologist and assistant professor of surgery. Results of the study are reported in the current issue of the journal *Clinical Cancer Research*.

The current standard to screen for prostate cancer is the PSA (prostate-specific antigen) blood test. Botchkina and other experts complain that the test is notoriously inaccurate, producing false positives at a relatively high rate.

As a result, men undergo invasive needle biopsies in search of cancers that do not exist. An elevated PSA also may be a sign of a harmless prostate condition known as benign prostatic hyperplasia, or it may signal an infection or inflammation of the prostate.

Telomerase appears in urine only when cancer has occurred, thus eliminating the possibility of benign conditions. Botchkina's telomerase study, which is sponsored by the U.S. Department of Defense, is one of many attempting to find more accurate methods of diagnosing common forms of cancer.

Prostate cancer is one of the most common malignancies in U.S. men. The American Cancer Society estimates that 230,000 cases of the disease will be diagnosed this year and about 30,000 men will die of the disease.

"It is really important to de-

velop new molecular tools so that we have more accurate diagnosis," Botchkina said.

The enzyme pinpointed in her studies normally governs the stability of "telomeres," the very tips of chromosomes. Think of a telomere as the protective encasement on the end of a shoelace.

Botchkina's work is just one in hundreds of investigations in worldwide racing to find new molecular "biomarkers," proteins and other infinitesimal compounds in blood or urine that can accurately signal prostate cancer's presence.

Dr. Arul Chinnaiyan, associate professor of urology at the University of Michigan, is part of a team that has gotten its prostate cancer screening into clinical testing at several major cancer centers. It is based on a protein called alpha-methylacyl coenzyme A, which can be spotted in blood when prostate cancer develops.

"There's a race on for diagnostic biomarkers, and there's also a race on to find biomarkers that can determine which [prostate] cancers are indolent and those that are aggressive," Chinnaiyan said.

## Fish mapped

Scientists say the variety of tuna, marlin, swordfish and other big ocean predators has declined up to 50 percent in the past half-century because of overfishing.

For the first time, ecologists and oceanographers mapped the hotspots with the largest concentrations of many big fish species, then and now. Their findings are reported in yesterday's online edition of the journal *Science*.

Researchers who had reported an overall decline in the abundance of big fish now say there also has been a significant drop in the variety of fish in many areas.

They did find five hotspots, said Boris Worm, a marine ecologist at Dalhousie University in Canada, the lead author. In the United States, two are in the Atlantic Ocean, east of Florida, and in the Pacific Ocean, south of Hawaii.

— THE ASSOCIATED PRESS

## Hypertension fact of aging

High blood pressure is defined as readings equal to or greater than 140, when the heart is beating, over greater than or equal to 90, when the heart is resting. Hypertension is a fact of aging for most people. Even if they don't have high blood

# Talk on doctors' role in Nazi regime

BY JAMIE TALAN  
STAFF WRITER

A Long Island meeting of members of the medical professions grappled yesterday with the issue of doctors, nurses and midwives working under the Nazi regime.

the way to Auschwitz," he said. "We should all recognize it."

Heberer agrees. "We would like to think medicine is a force for healing, but these traditional concepts were profoundly shaken in the 1930s."

At the time Germany was





## Stony Brook Researcher Identifies a Potential New Way to Diagnose Prostate Cancer

A Stony Brook University researcher may be on the path to developing a non-invasive and more specific diagnostic tool for prostate cancer based on studies focusing on telomerase, an enzyme known to be inactive in the majority of normal cells, but active in cancer cells. The results of the research on telomerase activity in the urine of patients with prostate cancer and men with benign prostatic hyperplasia could one day lead to a simple urinalysis test for the disease. The method involves a quantitative analysis of telomerase, an enzyme that directs the replication of telomeres at the ends of chromosomes. This enzyme is inactive in normal cells, but active in cancer cells.

Galina Botchkina, PhD, assistant professor in the Department of Surgery at Stony Brook University, and her colleagues analyzed the exfoliated cells from the urine of 56 patients. They

reported that 100 percent of those with confirmed prostate cancer had high telomerase activity. In contrast, 70 percent of those with benign prostatic hyperplasia did not express any telomerase activity.

"The research tells us that there is a potential new way to diagnose prostate cancer that will be more reliable than currently exists," says Dr. Botchkina, whose findings have been reported in *Clinical Cancer Research*. "Based on what we now know, the ultimate goal of a simple urinalysis test for prostate cancer is certainly possible."

Although telomerase activity alone has not yet been proved as an independent marker for prostate cancer, Dr. Botchkina believes the evidence is strong enough to suggest that quantitative analysis of telomerase activity may be used for early, non-invasive detection of prostate cancer. She also

believes that levels of telomerase activity, in combination with other molecular markers, such as metalloproteinases and adhesion molecules, can be used to determine tumor aggressiveness and its future behavior, thereby helping to avoid unnecessary aggressive treatment of slowly developing tumors and insufficient treatment of aggressive ones.

Prostate cancer is the most common type of cancer found in American men, other than skin cancer, with over 230,000 new cases in the U.S. alone last year, according to the American Cancer Society, and prostate cancer is the second leading cause of cancer death in men, second only to lung cancer. However, recent research indicates that prostate specific antigen (PSA) testing, the current standard diagnostic method, can be unreliable, underscoring the need for more sensitive and accurate testing.

## Stony Brook Research Sheds New Light on Mediators for Alzheimer's Disease

New research that includes information based on studies conducted by William Van Nostrand, PhD, professor of medicine at Stony Brook University, gives scientists a new focus in the search for the cause of Alzheimer's disease.

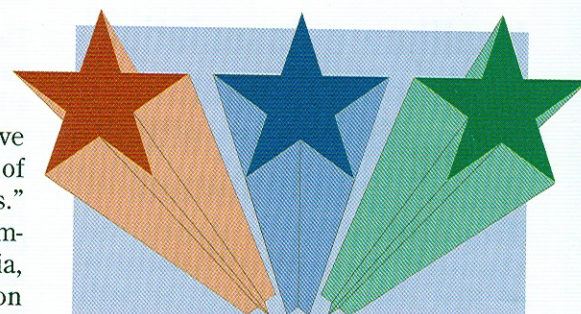
The research suggests that accumulation of amyloid beta peptide in cerebral blood vessels, as opposed to the brain itself, may be a more important pathological mediator of Alzheimer's disease. Two independent yet related articles describe such findings in the August issue of the *American Journal of Pathology*. Dr. Van Nostrand is co-author of the first study.

"These studies show us that it is important to be able to clear amyloid beta peptides from the brain into the blood to prevent the accumulation that occurs in Alzheimer's disease," says Dr. Van Nostrand. "When the amyloid beta peptides cannot get out of the brain and accumulate in brain vessels, damage to the vessels can result contributing to cognitive impairment. This knowledge will be critical as we continue to develop

therapeutic agents to treat cognitive impairment caused by this type of damage to brain blood vessels." Alzheimer's disease is the most common form of progressive dementia, affecting an estimated 4.5 million Americans, according to the Alzheimer's Association. Research that may lead to new therapies and prove beneficial offers hope to patients with Alzheimer's, and to their loved ones.

The second article, by Kumar-Singh *et al.*, "Dense-core plaques in Tg2576 and PSAPP mouse models of Alzheimer's disease are centered on vessel walls," uses two different transgenic mice: Tg2576 and PSAPP. Both models produce dense-core plaques, highly concentrated deposits of amyloid beta peptide, and were used to investigate the possible association of blood vessels with deposits.

These studies describe several animal models for further examining the pathogenesis and treatment of Alzheimer's disease and related cerebral amyloid angiopathies.



### Electronic Patient Record

#### STARS

The Cerner electronic patient record (PowerChart) is now named STARS, which stands for Stony Brook's Totally Automated Record System.

Congratulations to Perilynn Baldelli of Nursing Quality Management, the winner of the contest to name the new system.

Classes for STARS are being conducted in the IT training room in the HSC library. The class schedule is posted on the EPR website which is on the hospital's Intranet. If a class session is not an option for your staff, a web-based training link on the EPR site is also available. Classes are open to all departments; please call 4.5800 to reserve a seat.